THE EFFECT OF INFECTION WITH PSEUDORABIES VIRUS ON LOW MOLECULAR WEIGHT RNAS IN BHK 21/13 CELLS.

by Wilma M. Shepherd

Summary.

A general review of the mechanism of translation and a description of the essential components of the translation system are presented. Evidence of changes induced by bacteriophage and animal virus infection at a variety of different points in the host cell translation mechanism are described and the implications of such changes discussed on a theoretical basis. The effect of infection of viruses of the Herpes group on the population of low molecular weight RNAs in mammalian cells is considered in more detail.

Reported in this thesis are investigations designed to compare low molecular weight RNAs in non-infected and pseudorabies virus infected BHK 21/13 cells. The three separate approaches used and the results obtained are briefly described below.

Molecular hybridisation experiments are reported which indicate that a proportion of the 4s RNA synthesised in pseudorabies virus infect

cells, which possesses all the characteristics of tRNA, is specified by the viral genome. The material occupies 0.15% of the viral DNA and corresponds, at most, to four tRNA molecules per DNA. This quantitation is however only an approximation and is subject to several limitations which are discussed.

The populations of certain aminoacyl-tRNAs from non-infected and virus infected cells are compared using standard chromatographic techniques such as MAK column chromatography and Reverse phase type chromatography of aminoacyl-tRNA, and DEAE cellulose chromatograph of T_1 RNase digestion products of aminoacyl-tRNA. In all experiments the preparations from host and virus infected cells are differentially labelled in the amino acid moeity, mixed and subjected to co-chromatog to provide a valid comparison. The separation patterns of arginyl-tRN and lysyl-tRNA on MAK column chromatography, of threonyl-tRNA, seryl-tRNA and arginyl-tRNA on Reverse phase type II chromatography a of arginyl, lysyl, seryl and alanyl oligonucleotides on DEAE cellulose chromatography, are shown. In all cases, the distribution patterns of the $[^3H]$ and $[^{14}C]$ labels are superimposable and indicate neither the prese of any new or modified species of tRNA nor any alterations in the balanc of the host tRNA populations.

Evidence is then presented, that from as early as 2 hours post infection, there exists in the cytoplasm of pseudorabies infected cells, pulsed for 30 minutes with [^{3}H]uridine-5-[redacted] T, a species of RNA not detected in similarly labelled non-infected cultures. The RNA, termed $4\frac{1}{2}$ s RNA, occupies a larger molecular volume on Sephadex G-100 than cellular RNA and does not appear to contain methyl groups, derived from [^{14}C]methyl methionine. At least in part, it may be converted to material with characteristics 4s RNA properties by incubation of the cells in the absence of label for a further 60 minutes, or by incubation in vitro with a crude extract of non-infected but not infected cells. The $4\frac{1}{2}$ s RNA probably has a more open configuration than the conventional cloverleaf and may be similar to pre-tRNAs in Krebs II ascites tumour cells. However, conversion of $4\frac{1}{2}$ sRNA to 4sRNA by site binding of Mg^{2+} has not been demonstrated to occur. This RNA species is tentatively identified as an intermediate in the production of mature 4s RNA and hypotheses concerning its origin and function are discussed.

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ABBREVIATIONS

In addition to the accepted standard abbreviations, the following were used.

mRNA	=	messenger RNA
sRNA	=	soluble RNA
tRNA	=	transfer RNA
$tRNA^{leu.}$	=	transfer RNA for leucine
DNase	=	deoxyribonuclease
RNase	=	pancreatic ribonuclease
RNase T_1 T_1 RNase	=	ribonuclease I from Takadiastase
SDS	=	sodium dodecyl sulphate
MAK	=	methylated albumin Kieselguhr
U. V.	=	ultraviolet
P. I.	=	post infection
P. F. U.	=	plaque forming units
dpm	=	disintegrations per minute.

ERRATA

i) p. 145 follows p. 143

ii) for Sherberg p. 57 - read Scherberg.

iii) for Hoageland p. 91 - read Hoagland.

INTRODUCTION

INTRODUCTION

In any living cell there are two main classes of chainlike molecules, the nucleic acids and the proteins. The nucleic acids serve to provide the cellular genetic information (DNA) and to mediate in its accurate translation (RNAs) initially into polypeptides and thence into the protein molecules required to fulfil the normal functions of the cell. Such functions include replication, synthesis and repair of structural components, uptake and utilization of molecules available in the environment and degradation to and elimination of waste products etc.

PROTEIN SYNTHESIS

The first stage in the sequence of events leading to protein synthesis - transcription - involves enzymatically copying a structural gene (segment of DNA in which information for protein synthesis is stored) to form a single stranded RNA molecule of analogous base sequence to one of the strands of DNA. This component is termed messenger RNA since inherent in its base sequence is the information, or message, necessary to direct polypeptide synthesis during the second, or translational, stage. In many viruses, the genetic material is simply RNA

Table 1.

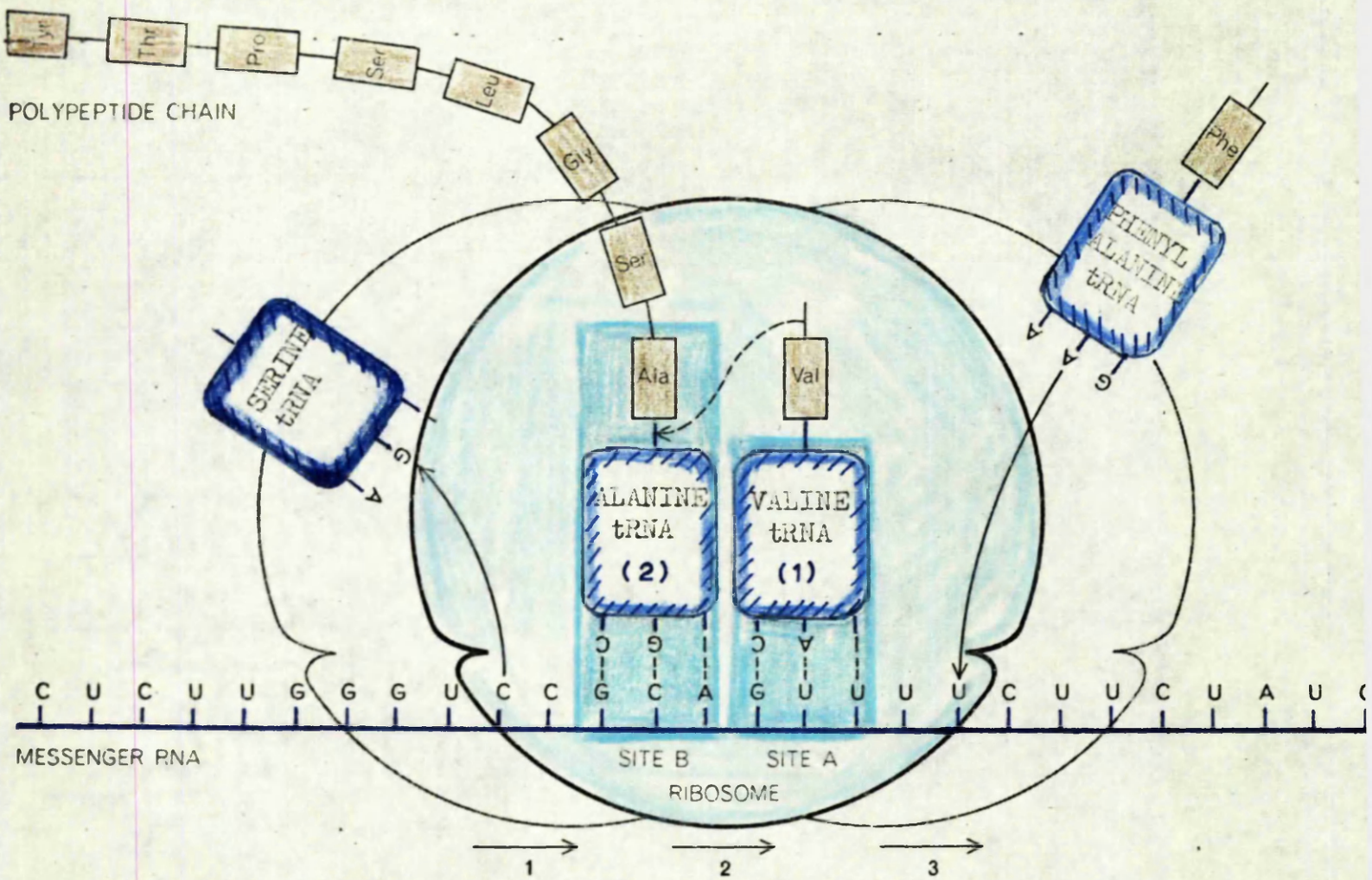
FIRST LETTER	SECOND LETTER: U	SECOND LETTER: C	SECOND LETTER: A	SECOND LETTER: G	THIRD LETTER
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA OCHRE	UGA ?	A
	UUG Leu	UCG Ser	UAG AMBER	UGG Tryp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA GluN	CGA Arg	A
	CUG Leu	CCG Pro	CAG GluN	CGG Arg	G
A	AUU Ileu	ACU Thr	AAU AspN	AGU Ser	U
	AUC Ileu	ACC Thr	AAC AspN	AGC Ser	C
	AUA Ileu	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

GENETIC CODE, consisting of 64 triplet combinations and their corresponding amino acids, is shown in its most likely version. The importance of the first two letters in each triplet is readily apparent. Some of the allocations are still not completely certain, particularly for organisms other than the colon bacillus (*Escherichia coli*). "Amber" and "ochre" are terms that referred originally to certain mutant strains of bacteria. They designate two triplets, UAA and UAG, that may act as signals for terminating polypeptide chains.

which itself acts as a messenger. It appears that reading of the message commences at the 5' end of the polynucleotide and that polypeptides are synthesised from the free amino end. The message is embodied in the sequence of units of 3 nucleotide bases (or codewords) each of which denotes (with 3 exceptions) a specific amino acid. The relationship between codewords and amino acids is known as the genetic code and is shown in Table I (reproduced from "The Genetical Code III" Crick (1966a)). The "code" used is a degenerate one, there being two or more codewords, for most of the amino acids. Further discussion of the genetic code and its implications are deferred for the present.

The process of protein synthesis is not dependent on direct steric interactions between the trinucleotides and the amino acids. Instead, specific intermediates - tRNAs - which serve as adaptors (Crick, 1958) between the mRNA and the appropriate amino acids, are required (von Ehrenstein, Weisblum & Benzer, 1963; Chapeville, Lipmann, von Ehrenstein, Weisblum, Ray & Benzer, 1962). The rigid specificity of the process is, therefore, embodied in the tRNA molecules which normally attach, by enzymically mediated reactions, only the requisite amino acid to the growing peptide chain. (A diagrammatic representation is

Figure 1.



SYNTHESIS OF PROTEIN MOLECULES is accomplished by the intracellular particles called ribosomes. The coded instructions for making the protein molecule are carried to the ribosome by a form of ribonucleic acid (RNA) known as "messenger" RNA. The RNA code "letters" are four bases: uracil (U), cytosine (C), adenine (A) and guanine (G). A sequence of three bases, called a codon, is required to specify each of the 20 kinds of amino acid, identified here by their abbreviations. (A list of the 20 amino acids and their abbreviations appears on the next page.) When linked end to end, these amino acids form the polypeptide chains of which proteins ar composed. Each type of amino acid is transported to the ribosom by a particular form of "transfer" RNA (tRNA), which carries a anticodon that can form a temporary bond with one of the codon in messenger RNA. Here the ribosome is shown moving along th chain of messenger RNA, "reading off" the codons in sequence. I appears that the ribosome has two binding sites for molecules o tRNA: one site (*A*) for positioning a newly arrived tRNA mole cule and another (*B*) for holding the growing polypeptide chain

shown in Fig. 1).

Peptide formation occurs on complex cytoplasmic particles - the ribosomes, and requires, in addition to the messenger RNA, an assortment of species of aminoacyl-tRNAs and also various protein factors. The ribosome is assumed to function by maintaining all the components of the translation apparatus in the optimal steric configuration. At any one polymerisation step, the appropriate incoming aminoacyl-tRNA (-tRNA(1)), determined by the mRNA molecule, attaches to the ribosome at site A. Peptide bond formation between the amino acid attached to tRNA(1) and the polypeptide attached to tRNA(2) at site B, then occurs. tRNA(2) is released and the growing polypeptide chain, thus lengthened by 1 amino acid, is now attached to tRNA(1) which still occupies position A. The peptidyl-tRNA is then translocated to position B where it remains attached prior to the addition of a further amino acid.

The initiation signal for polypeptide synthesis, at least in bacteria, appears to be the triplet AUG (and perhaps GUG) which codes for formyl methionine at 5' termini (Clark & Marcker, 1966; Sundararajan & Thach, 1966) and also exhibits strong phase selector characteristics, thus maintaining the proper reading

frame within the message. The signal for chain termination appears to be effected by the triplets UGA, UAG and UAA (Last, Stanley, Salas, Hille, Wahba & Ochoa, 1967; Brenner, Stretton & Kaplan, 1965), although UAA is thought to be that normally utilised in vivo (Garen, Garen & Wilhelm, 1965).

By 1965, it had become generally accepted that when a virus successfully invaded a cell the virus specified proteins were synthesised by the unaltered translation mechanism of the cell. The levels of those components essential for protein synthesis were thought to be maintained in a manner similar to that in the normal uninfected cell. It was postulated that the only likely alteration after virus infection was one designed to induce preferential translation of virus specified mRNA.

Such an interpretation is no longer valid. Several different changes in the protein synthetic mechanism detected in virus infected cells have been directly related to the expression of the viral genome. The alterations observed, subsequent to both bacteriophage and animal virus infections, are described below. A more detailed review of normal protein synthesis and its role in cellular regulation is also presented, so that the nature and implications of the virus specified changes may be fully appreciated.

First the major components of the protein synthesising system are reviewed:

Transfer RNAs

tRNA molecules are essential components of the protein synthesising system and comprise a population heterogeneous with regard to amino acid attachment and codon recognition sites (termed anticodons) (Nirenberg, Matthaei & Jones, 1962). The tRNA molecules themselves are modified forms of RNA transcribed originally from cellular DNA genes (Giacomoni & Spiegelman, 1962; Goodman & Rich, 1962) which may map in the same region as ribosomal RNA e.g. in B. subtilis (Oishi, Oishi & Sueoka, 1966; Dubnau, Smith & Marmur, 1965), or show no correlation to it, as with Drosophila melanogaster (Ritossa, Atwood & Spiegelman, 1966). It is estimated from saturation studies in Drosophila (Ritossa, Atwood & Spiegelman, 1966) that 10 to 20 gene sites exist per tRNA molecule, assuming that there are approximately 60 tRNA species in the cell.

In general, all tRNAs have the following common characteristics. They have a molecular weight of approximately 25,000, sediment in the region of 4s RNA, are approximately 80 nucleotides long, possess a high G+C content, terminate at the 3' end with the

nucleotide sequence pCpCpA (Hecht, Stephenson & Zamecnik, 1959) and contain many structurally modified bases in their nucleotide sequence (see p.23,24). Addition of pCpCpA to the 3' OH end (Preiss, Dieckmann & Berg, 1961) and modification of the existing bases occurs enzymically to the already transcribed tRNA polynucleotide chain. The most commonly found unusual bases or nucleosides are pseudouridine (Cohn, 1960), dihydrouridine (Madison & Holley, 1965), inosine (Holley, Everett, Madison & Zamir, 1965), thionucleosides and most important methylated nucleosides. Other less common bases have also been detected (Robins, Hall & Thedford, 1967). Many modified bases normally occur near the 5' end of the molecule but a more detailed description of the known modifications follows.

Different tRNAs have different sequences and the heterogeneous nature of the tRNA population may be demonstrated by a variety of techniques such as Counter Current Distribution (Holley, Apgar, Doctor, Farrow, Marini & Merrill, 1961; Holley & Merrill, 1959); Sephadex-G25 chromatography (Muench & Berg, 1966a; Bergquist, Baguley, Robertson & Ralph, 1965); Reverse phase chromatography (Kelmers, Novelli & Stulberg, 1965; Weiss & Kelmers, 1967); MAK column chromatography (Mandell & Hershey, 1960;

Sueoka & Yamane, 1962); DEAE cellulose chromatography (Cherayil & Bock, 1965; Baguley, Bergquist & Ralph, 1965); Benzoylated DEAE cellulose chromatography (Gillam, Millward, Blew, von Tigerstrom, Wimmer & Tener, 1967; Roy & Söll, 1968) and Hydroxyapatite chromatography (Muench & Berg, 1966b; Pearson & Kelmers, 1966; Harding, Schauer & Hartmann, 1966), to mention but a few.

These methods not only result in the separation between different aminoacyl-tRNAs but also reveal that several types of tRNA exist for certain amino acids. The distribution patterns for the population of tRNAs vary from one organism to another and even between cell sap and mitochondria (Buck & Nass, 1968; Epler & Barnett, 1967).

The linear base sequence of several individual tRNA molecules from different sources, mammalian, fungal and bacterial, have been elucidated, after being extensively purified. Molecules sequenced include species specific for a variety of amino acids (Raj-Bhandary, Chang, Stuart, Faulkner, Hoskinson & Khorana, 1967; Holley, Apgar, Everett, Madison, Marquissee, Merrill, Penswick & Zamir, 1965; Reeves, Imura, Schwam, Weiss, Schulman & Chambers, 1968; Staehelin, Rogg, Baguley, Ginsberg

& Wehrli, 1968; Madison, Everett & Kung, 1966a; Baev, Venkstern, Mirzabekov, Krutilina, Li & Axelrod, 1967; Zachau, Dütting & Feldman, 1966; Goodman, Abelson, Landy, Brenner & Smith, 1968; Dube, Marcker, Clark & Cory, 1968; Doctor, Loebel, Sodd & Winter, 1969). Each sequence is unique both in linear sequence of the 4 common bases and in number, type and position of the modified bases. However, in all the tRNAs so far examined, the secondary structure imposed by intramolecular base pairing can be portrayed in terms of a cloverleaf model (Madison, Everett & Kung, 1966b; Zachau, Dütting, Feldman, Melchers & Karau, 1966) (illustrated for $tRNA^{tyr.}$ species in Fig. 2). That is to say, complementary regions form α helices and non-bonded regions "loop" out as single stranded stretches. Such single stranded regions are characterised by a high content of unusual bases, 55 out of the 67 known modified bases being so located. Loop IV generally contains a high percentage of dihydrouridine residues. In all cases, it is possible to locate in similar positions in loop III trinucleotide sequences, themselves often containing a modified base, which can be assigned to the appropriate anticodons. Although several other possible structures can be postulated

Figure 2.

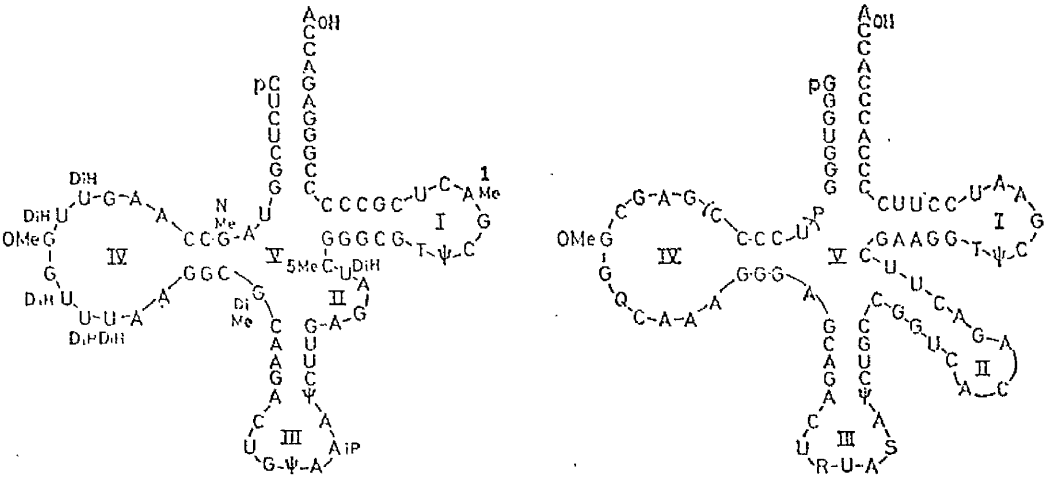


Fig. 2. Cloverleaf arrangement of the nucleotide sequences of yeast $tRNA^{Tyr}$ and *E. coli* $tRNA_2^{Tyr}$.

as illustrated in Doctor, Loebel, Sodd & Winter (1969)

from known sequence data, the cloverleaf model is considered most likely and is substantiated experimentally by measurements of physico-chemical parameters (Lake & Beeman, 1968).

tRNA molecules also possess a tertiary structure which perhaps reflects the preferred orientation and interaction of non-bonded regions within, or between, helical segments (Henley, Lindahl & Fresco, 1966). X-ray scattering methods appear to suggest that the cloverleaf configuration has 2 or 3 arms folded together and the remaining arm or arms extended in the opposite direction (Lake & Beeman, 1968). This tertiary structure appears to be less stable than the secondary one. Certain species of tRNA have been demonstrated to exist in two interconvertible conformational variations:- "native" and "denatured", say (Gartland & Sueoka, 1966; Muench, 1966; Fresco, Adams, Ascione, Henley & Lindahl, 1966). The "native" configuration is stabilised by Mg^{2+} and heating at 60° in the presence of Mg^{2+} for only a matter of minutes results in the conversion of the "denatured" to the "native" form (Lindahl, Adams & Fresco, 1966). Only the "native" configuration possesses the characteristic biological activities as regards aminoacylation reactions (Gartland & Sueoka, 1966; Fresco et al, 1966), specificity of

response in polynucleotide stimulated ribosomal binding assays (Ishida & Sueoka, 1967) and capacity to mediate amino acid incorporation into polypeptides (Lindahl, Adams, Geroch & Fresco, 1967). The conformational change does not appear to result in the alteration of the tRNA species in a manner such as would enable it to recognize another codeword. If, therefore, as has been suggested, the inactive form is attributable to changes resulting in a different trinucleotide sequence occupying the anticodon position in loop III (the slippage hypothesis (Ishida & Sueoka, 1967)) then the substitute triplet is not recognized as amino acid specifying.

A further elaboration of tRNA configuration, termed quaternary, has been described by Loehr & Keller (1968). This quaternary structure appears to be more stable than the corresponding secondary and tertiary ones and is thought to be a double cloverleaf, i. e. to consist of 2 single tRNA cloverleafs covalently attached through the anticodon loops. The dimers are capable of accepting 2 molecules of the same amino acid at the same rate as monomers but, as might be expected, are unable to participate in codon recognition. No biological significance has as yet been determined for the dimers.

Genetic Code

The genetic code, as shown in Table I, has been elucidated mainly using the ribosomal binding assay in response to certain trinucleotides (Nirenberg & Leder, 1964), in vitro amino acid incorporation stimulated by polynucleotides of random or specifically determined base sequences (Khorana, 1965) and by correlation of amino acid substitutions with known genetic mutations (Yankofsky, Ito & Horn, 1966; Streisinger, Okada, Emrich, Newton, Tsugita, Terzaghi & Inouyl, 1966). This code appears to be universal and applies equally well to mammalian, plant, bacterial and viral systems (von Ehrenstein & Lipmann, 1961; Tsugita, Fraenkel-Conrat, Nirenberg & Matthaei, 1962; Weinstein & Schechter, 1962). Since more than 1 codeword is designated for all the amino acids except tryptophan and methionine, the code is termed degenerate. Indications at present suggest that the synonym codons are recognized by different tRNA molecules specific for the same amino acid (iso-accepting tRNAs) (von Ehrenstein & Dais, 1963; Weisblum, Gonano, von Ehrenstein & Benzer, 1965; Kellogg, Doctor, Loebel & Nirenberg, 1966; Galizzi, 1967). Such correlation can be demonstrated directly using in vitro synthesis of rabbit haemoglobin (Weisblum, Cherayil,

Bock & Söll, 1967). In this system, the arginine bound to different species of $tRNA^{arg.}$, say, is not incorporated randomly into the positions in the polypeptide chain normally occupied by this amino acid. Instead, each arginyl-tRNA species specifically donates its amino acid to only certain positions in the haemoglobin chain. By utilising substitution data, these sites can be shown to be comprised of different arginine specifying codons. In vitro, obvious differences in the coding response of several aminoacyl-tRNAs have been observed, ranging from those in which a tRNA can only recognize one codon to those in which one tRNA can recognize up to 3 codons (Söll, Cherayil & Bock, 1967). Neglecting possible failure to separate highly similar tRNAs, it can be inferred, from the data available, that changes in the first letter of the codon generally require a new tRNA species (not formyl methionine). So does a guanine residue occupying the third position. A tRNA species is capable of multiple codon recognition when certain alterations in the third base are involved. In this case 2 further subdivisions are observed. Namely, these are that recognition of three codons by one tRNA involves classing U, C and A in a group and that recognition of two codons by one tRNA involves classing both pyrimidine or both purine bases together. Such observations are consistent with

the Wobble Hypothesis (Crick, 1966b).

Redundancy

The need for all the multiple tRNA species, however, cannot always be attributed just to the degenerate nature of the genetic code, since, the number of tRNA species for certain amino acids exceeds the number of codons assigned to them. When more than one tRNA species recognizes the same codon or codons, the 'extra' tRNAs are said to be redundant. The lysine system in yeast (Söll & Raj-Bhandary, 1967), the tyrosine suppressor system in E. coli (Goodman et al, 1968) and the seryl-tRNAs sequenced by Zachau (Zachau, Dütting & Feldman, 1966) all show evidence of redundancy. The apparent redundancy of the seryl-tRNAs mentioned may be attributable to a heterogeneity in the yeast used as starting material.

It should be noted that redundancy in more highly developed cells may be attributable to compartmentation of protein synthesis, e.g. in the mitochondria (Barnett & Brown, 1967; Epler & Barnett, 1967; Suyama & Eyer, 1967). Some tRNAs recognizing the same triplet may not, however, be indispensable for correct template readout. There are indications also that apparently redundant tRNAs may be used for special purposes. For instance,

the tRNA population in E. coli contains two tRNA species which differ only in two nucleotides, not in the anticodon, but only one of which can apparently mutate to a suppressor (Goodman et al, 1968). In S. aureus (Bumsted, Dahl, Söll & Strominger, 1968) three types of tRNA attach glycine and are active in peptidoglycan synthesis but only two of these have been shown to partake in protein synthesis. A similar pattern with seryl-tRNA has been reported in S. epidermis (Petit, Strominger & Söll, 1968) and it is possible to imply that, perhaps, a unique species of $tRNA^{gly.}$ (or $tRNA^{ser.}$) exists which is only functional in peptidoglycan synthesis.

These species may, of course, be functioning as suppressors. A form of $tRNA^{leu.}$ which does not become ribosome associated (Wettstein, 1966) and may have a regulatory role, also occurs (Yegian & Stent, 1969b). Certain tRNAs perhaps, therefore, perform functions unassociated with protein synthesis. At any rate, a regulatory role for tRNAs based on codon frequency and distribution may be postulated and several such systems will be discussed later.

Aminoacyl-tRNA Transferases

Aminoacyl-tRNA transferases have been isolated from animal

(Hoagland, Keller & Zamecnik, 1956), plant (Clark, 1958) and bacterial sources (DeMoss & Novelli, 1956). The enzymes catalyse both the activation of a specific amino acid and its transfer to a specified sRNA molecule. The reaction consists of 2 stages and can be represented as

Enz. + ATP + amino acid = Enz. - AMP - amino acid + PPi (1)

Enz. - AMP - amino acid + sRNA = Aminoacyl-tRNA + AMP + Enz. (2)

It therefore follows, that for each amino acid there exists an aminoacyl-tRNA transferase which normally catalyses the attachment of that amino acid to only those tRNA species which recognize the codons allotted to it. It is unclear, at present, whether one transferase is required for each iso-accepting tRNA and whether inter-species homology is exhibited by the transferases, but both topics will be reviewed later.

Purified aminoacyl-tRNA transferases have molecular weights of approximately 100,000, with the exception of phenylalanyl-tRNA transferase (Makman & Cantoni, 1965; Stulberg, 1967), and most appear to have about 8 sulphydryl groups per molecule (DeLuca & McElroy, 1965; Stern, DeLuca, Mehler, & McElroy, 1966). As isolated they are strongly attached to nucleic acid and may remain linked to it through

several purification steps (Seifert, Nass & Zillig, 1968; Allende, Allende, Gatica, Celis, Mora & Matamala, 1966). Indeed, this attachment, in certain cases, may serve to stabilise the enzyme (Papas & Mehler, 1968).

The specificity of amino acid selection is introduced at the level of reaction 2 (Arca, Frontali, Sapora & Tecce, 1967; Yarus & Berg, 1967), since transferases may, in the absence of the correct amino acid, anomalously bind amino acids of similar structure to the appropriate molecule but are unable, except in the cases of high amino acid analogy, e. g. canavanine and arginine (Mitra & Mehler, 1967), to effect the transfer of the wrong amino acid to the tRNA. To maintain the necessary degree of selectivity, the overall binding of the amino acid and the tRNA to the enzyme is thought to be the result of a large number of weak interactions and to be subject to strict stereochemical restraints (Loftfield & Eigner, 1967). Only correct pairing between an amino acid and transferase results in a configurational change in the enzyme (Ohta, Shimada & Imahori, 1967) and some amino acids are only transferred to tRNA molecules in particular stereochemical configurations (Gartland & Sueoka, 1966; Lindahl et al, 1967).

These enzyme reactions occur at pH 7.0-8.0 and require Mg^{2+} and ATP for activity (Novelli, 1967; Allende, Mora, Gatica & Allende, 1965). The actual pH optimum and the optimal ratio of Mg^{2+} concentration to ATP concentration are different for each transferase, both within and between species. For instance, E. coli leucyl-tRNA transferase has an optimal ratio of 10 to 1 whereas that of prolyl-tRNA transferase, derived from the same organism, is 30 to 1 (Novelli, 1967). Also, the optimal ratio of operation differs between cytoplasmic and mitochondrial aminoacyl-tRNA transferases derived from the same source (Lietman, 1968). In certain cases the concentration of monovalent ions, especially ammonium and potassium, have been shown to be important enzyme stimulators (Yu & Hirsh, 1967; Loftfield & Eigner, 1967; Holley, Brunngraber, Saad & Williams, 1961). Other transferases do not demonstrate any dependence on univalent ions and Na^{+} may act as an inhibitor of esterification (Peterkofsky, Gee & Jesensky, 1966). The salt effect appears to depend on interactions which may act by stabilising the enzyme - tRNA complex.

Several observations indicate that the esterification reaction depends both on the source of the tRNA and of the transferase.

Various degrees of interchangeability of the tRNA and the transferase between different organisms occur, the degree depending on the organism from which the enzyme and the tRNA are isolated and also the identity of the amino acid under consideration. The four responses observed are outlined below. In certain cases, no cross reaction is observed when the enzyme and the tRNA are derived from separate sources (heterologous reaction), (Hayashi, 1966; Loftfield & Eigner, 1963; Yamane, Cheng & Sueoka, 1963). The nucleotide sequences for enzyme recognition, therefore, are either not present or else the tRNAs have steric configurations which prevent the enzyme reacting with the heterologous RNA. But many heterologous reactions work and result in the esterification, both qualitatively and quantitatively, of the same tRNA species as in a homologous reaction (Yamane & Sueoka, 1963). Other heterologous esterification reactions result in the attachment of an amino acid to tRNA at a lower level of overall efficiency. e. g. with E. coli sRNA and yeast methionyl-tRNA transferase only a part of the methionine acceptor RNA can be charged (Berg, Bergmann, Ofengand & Dieckmann, 1961).

In a very few instances, heterologous reactions lead to the

aminoacylation of tRNA species not normally loaded with the amino acid in question by the homologous transferase. The leucyl-tRNA formed when yeast leucyl-tRNA formation is catalysed by E. coli transferase (about 1% of the available $tRNA^{leu.}$) is neither similar to that of normal yeast leucyl-tRNA, nor to E. coli leucyl-tRNAs (Yamane & Sueoka, 1963). A much more significant novel component is obtained from a cross-reaction using Neurospora crassa transferase to load E. coli tRNA with phenylalanine (Barnett, 1965). However, this reaction can now be attributed to contamination with mitochondrial tRNAs and transferases (Barnett, Brown & Epler, 1967; Barnett & Brown, 1967), and therefore, may be included in one of the previous categories (on the assumption that mitochondria differ in origin from the cell type in question).

In general in bacterial systems, only one transferase is required to catalyse the addition of any particular amino acid to all the iso-accepting tRNAs for that amino acid (Niyomporn, Dahl & Strominger, 1968; Yamane & Sueoka, 1963; Eidlic & Neidhardt, 1965; Nass & Neidhardt, 1967). Sundharadas, Katze, Söll, Konigsberg & Lengyel (1968) have demonstrated that one transferase catalysed the in vitro attachment of serine to both $tRNA\ ser^{IIa}$

and tRNA ser^{IIb} even though type IIa recognizes the codons AGU and AGC while type IIb recognizes UCA and UCG. However, Yu (1966) has reported the separation, by hydroxyapatite chromatography, of 3 'types' of leucyl-tRNA transferase from extracts of E. coli, the 2 minor peaks representing only 3% of the enzymic activity under normal conditions (Yu, 1966). In addition to the chromatographic separation, the enzymes show differences in response to ionic conditions and appear to have varying specificities towards certain of the $tRNA^{leu.}$ species. The chromatographic separation is not stable and on rechromatography of peak 2 material the original 3 peak pattern is obtained. It is, therefore, extremely likely that the different peaks equate with different sized aggregates of a common subunit.

There have, however, been several reports in higher organisms of two or more chromatographically distinct transferases which catalyse the addition of the same amino acid and are derived from the same source (Allende & Allende, 1964; Allende et al, 1966; Lagerkvist & Waldenström, 1967). For example, leucyl-tRNA transferase activity in the rat liver system elutes from hydroxyapatite in two distinct bands (Vescia, 1967). These 'enzymes' have different Km values and exhibit differences in

heat stability. Three leucyl-tRNA transferase fractions (Strehler, Hendley & Hirsch, 1967) which have been isolated from rabbit heart by DEAE cellulose chromatography, as in the case of E. coli, have different affinities for the individual species of $tRNA^{leu}$.

The appearance of these chromatographically distinguishable transferases can be explained in three ways.

(1) The chromatographic separation is genuine and different enzyme species are present.

(2) The activity peaks are different sized aggregates of a common monomeric unit.

(3) The observations are due to an artefact introduced by non-homogeneous starting material or compartmentation within the cell (Barnett & Epler, 1966).

It should also be noted that there are reports of variations in transferase activity between cells of identical genotype but different phenotype (Strehler, Hendley & Hirsch, 1967; Taylor, Granger, Buck & Holland, 1967).

In attempts to localize the site of transferase attachment to tRNA several approaches have been used. These include

comparing the esterification of normal tRNAs with chemically (Lindahl, 1967; Harriman & Zachau, 1966; Wade, Yamamoto, Fukutome & Kawade, 1968; Cerutti, 1968; DeVries, 1967) or genetically (Smith, 1969) modified ones or correlating the efficiency of inhibition of amino acid acceptance with the structure of inhibitors (Letendre, Michelson & Grunberg-Manago, 1966; Hayashi & Miura, 1966). The results reported have often been at variance and, so far, no definite determination of the attachment site has been ascertained. The anticodon itself would not appear to be involved in the main binding location (Penswick, 1966). The most favoured locations of the enzyme attachment site are in the loop nearest to the 5' end of tRNA molecules or in the second small loop from the 3' end (Doctor et al. 1969) or perhaps by combination of these two sites (Madison, Everett & Kung, 1966b). However, evidence in the tyrosine suppressor system indicates that the stair of the anticodon loop is involved (Smith, 1969) and Schulman & Chambers (1968) suggest, on the basis of U. V. photolysis data, that the first three base pairs from the 3' end are the recognition point for transferases but that the entire stem region is required to maintain the integrity of the binding site.

Thiolation

tRNA is known to contain as minor components, thionucleosides including 4-thiouridine (Lipsett, 1965), 2-thiocytosine, 5-methylaminomethyl-2-thiouracil (Carbon, David & Studier, 1968), 2-methylthio, 6N (γ, γ dimethylallyl) adenylic acid (Burrows, Armstrong, Skoog, Hecht, Boyle, Leonard & Occolowitz, 1968) and others as yet unidentified. The thiolations occur as additions to the common bases in the polynucleotide sRNA precursors (Lipsett & Peterkofsky, 1966). The enzymic reaction requires ATP, Mg^{2+} ions and pyridoxal phosphate to achieve the transfer of sulphur from cystine or cysteine into sRNA. The sulphur atom in 4-thiouridine is incorporated into uridine and, therefore, does not alter base pairing arrangements. Its presence, therefore, affects molecular stability but not configuration.

Ribosomal RNA or synthetic polynucleotides cannot be substituted for sRNA, perhaps because of the secondary structure of the latter (Hayward & Weiss, 1966). Normal E. coli sRNA, although containing its full complement of sulphur molecules, can act as a sulphur receiver in vitro. Rabbit liver and yeast sRNAs, which are thought to be naturally deficient in sulphur atoms, are reported not to accept sulphur in the system devised by Lipsett

& Peterkofsky (1966) while yeast and rat liver sRNAs act as acceptors in that of Hayward & Weiss (1966).

Methylation

sRNA usually contains methylated purine and pyrimidine nucleosides in addition to the 4 normally found. At least 19 modified species have been identified but this may still be an underestimate. The methyl grouping may either be introduced into the N heterocyclic rings of the bases, into the glycosyl parts of the residue (Nichols & Lane, 1966) or even into both positions. Incorporation occurs at the macro-molecular level and is catalysed by a number of highly specific enzymes (Hurwitz, Anders, Gold & Smith, 1965). The methyl groups are normally transferred from S adenosyl methionine to sRNA.

The introduction of methyl groups into tRNA causes disturbances in the steric and electronic patterns and thus alters both the configuration and electron density distribution of the molecules. The precise effect is determined by the position occupied by the methyl grouping (Littauer, Revel & Stern, 1966). For instance, formation of 5-methyl uridine enhances base pairing in that region while substitution of a methyl group at the 3N position of uridine or methylation of 2' OH of ribose leads to

a decrease in secondary structure.

The general properties of the enzyme catalysing modifications of this type - methylases - are as follows. The methylases, in mouse and rat systems, appear to have a pH optimum of 8.5-9.0 for the methylation of all bases (Kaye & Leboy, 1968; Rodeh, Feldman & Littauer, 1967). Both systems are stimulated by NH_4^+ ions, the rate of incorporation being increased 3-5 fold. In rat liver, the rate of methyl incorporation into all four target bases was increased to the same extent. Using the mouse system, NH_4^+ stimulation led to an alteration of the pattern of methylated bases introduced. The enzymes appear to operate maximally at 42° and indeed, activity may be observed for certain enzymes at 48°.

The study of the role of methylated bases in tRNA molecules has been examined using species of tRNA which accumulate in a relaxed methionine requiring strain of E. coli during methionine starvation (Mandel & Borek, 1961). The proportion of this RNA increases with increasing deprivation time and can, indeed, be shown to be deficient in methyl groups. It can be separated chromatographically from its methylated counterparts, at least

in the case of phenylalanyl-tRNA on MAK (Revel & Littauer, 1965) and leucyl-tRNA on Reverse phase chromatography (Capra & Peterkofsky, 1968) and on MAK (Lazzarini & Peterkofsky, 1965). Neither the aminoacylation of such methyl deficient species of tRNA (Littauer, Muench, Berg, Gilbert & Spahr, 1963; Peterkofsky, Jesensky, Bank & Mehler, 1964), the attachment of aminoacyl-tRNA (under-methylated) to polynucleotide/ribosomal complexes (Lazzarini & Peterkofsky, 1965), nor the subsequent amino acid transfer (Littauer et al, 1963; Peterkofsky et al, 1964) appear to differ significantly from normal. It should, however, be noted that Shugart, Novelli & Stulberg (1968) report that the aminoacylation reaction with methyl-deficient tRNA is depressed when compared to normal, the degree of depression depending on the degree to which methylation is absent. However, the under-methylated condition of the tRNA moeity does lead to the alteration of the coding responses of $tRNA^{leu.}$ (Capra & Peterkofsky, 1966; Capra & Peterkofsky, 1968) and $tRNA^{phe.}$ (Revel & Littauer, 1966).

Phenylalanyl-tRNA (under-methylated) is capable of recognising codons other than those normally associated with phenylalanine (UUU, UUC). The miscoding occurs even under optimal

translation conditions. The leucine system provides no evidence that miscoding takes place with methyl deficient tRNAs. In this system, evidence obtained using poly UG and poly UC binding as criteria indicates that methylated species and their submethylated counterparts respond to different leucine codewords e.g., a chromatographically distinct species of leucyl-tRNA which appears after methionine deprivation exhibits poly UC binding instead of binding both poly UC and poly UG.

After in vitro methylation, the chromatographic and translation properties of the RNAs, with one exception, return to normal (Shugart, Chastain, Novelli & Stulberg, 1968; Capra & Peterkofsky, 1968). This exception involves a species of leucyl-tRNA which cannot be converted to normal by in vitro methylation or else accepts the methyl groups but still retains the old configuration.

So far, it has not proved possible to elucidate the function of methylated bases in tRNA structure, although a specific stretch of nucleotides (TψUpCpGp) is thought to be involved in attachment of the tRNA to the ribosomes (Sarkar & Comb, 1966). Certainly, the more complex modified base 2-methylthio, 6N (γ, γ dimethylallyl) adenylic acid is involved in ribosomal binding (Gefter & Russell, 1969).

Ribosomes

Little is known concerning either the detailed structure of the ribosomes or exactly how they function. The particles are complex ones containing RNA and protein and have diameters of 140Å - 180Å. They are composed of two unequal subunits - see Fig. 1. Each subunit contains a variety of protein components and at least one major characteristic RNA molecule. A further structural RNA, termed 5s, is also associated with the larger subunit. The ribosomes as has already been shown play a vital part in protein synthesis, acting as the attachment site for the various components involved. Normally, more than one ribosome is attached to a mRNA at any one time. These groupings are termed polysomes.

5s RNA

A low molecular weight species of RNA, distinct from tRNA (Rosset & Monier, 1963; Galibert, Larsen, Lelong & Boiron, 1965) has been described in most cell types. This RNA is termed 5s RNA. The molecule is 120 nucleotides long, contains no unusual bases (Brownlee, Sanger & Barrell, 1967; Forget & Weissman, 1967b) and does not accept amino acids (Rossett, Monier & Julien, 1964; Galibert, Larsen, Lelong & Boiron, 1966). Comparisons of primary sequences or of fingerprint maps of 5s RNA from different cell types show that the sequence of each species of 5s RNA, though mostly corresponding, does contain characteristic sequences. Configurations of the molecules, based on the reported sequences, melting profiles etc., show a degree of

of similarity with tRNA. They are composed of long regions of base pairing (60-70% of bases in E. coli) and single stranded nucleotide loops (Raacke, 1968; Boedtker & Kelling, 1967; Cantor, 1968). In both cases, (5s and tRNA) the structures are Mg^{2+} stabilised (Comb & Zehavi-Willner, 1967). It has been suggested (Sarkar & Comb, 1969) that the configuration of 5s RNA determined in vitro does not correspond to the in vivo functional form.

The largest portion (98%) of 5s RNA molecules in uninfected cell systems is found associated with the ribosomal fraction. This species of RNA appears to be a stable, fundamental component of the ribosomes (Watson & Ralph, 1967; Kaempfer & Meselson, 1968; Sarkar & Comb, 1969) and to be distinct from larger ribosomal RNAs. One 5s RNA molecule is associated with one 50s subunit and the 30s subunit contains no 5s RNA (Siddiqui & Hosokawa, 1968; Morrell and Marmur, 1968). The exact location of 5s RNA within the 50s subunit is not, however, known.

Several theories for 5s RNA function have been postulated. These suggest that perhaps 5s RNA may act to prevent peptide linkages between adjacent proteins (Brenner, Stretton & Kaplan, 1965), that it may bind nascent polypeptides to the 50s subunit (Kuriki & Kaji, 1966) or that it may function in ribosomal assembly.

The origin of 5s RNA has not yet been unequivocally determined. Based on hybridisation results in B. subtilis, it appears that 5s RNA is synthesised as a distinct species of ribosomal RNA since it maps separately from, but in the same genetic region as, other ribosomal RNAs and tRNA (Smith, Dubnau, Morrell & Marmur, 1968). Further evidence for 5s RNA forming a separate constituent ribosomal RNA is obtained by kinetic studies (Galibert, LeLong, Larsen & Boiron, 1967). However, it is not clear from these studies whether 5s RNA is formed as a bye-product during maturation of ribosomal 23s RNA (Hecht, Bleyman & Woese, 1968). In this respect, it should be noted that, although 23s RNA is methylated, precursor species are not.

Recently, other ribosome associated low molecular weight RNAs have been reported (Parish, Kirby & Klucis, 1966; Gardner & Hoagland, 1968; Watson & Ralph, 1966). The RNA species reported by Pene, Knight & Darnell (1968) and termed 7s RNA is thought to be formed from 32s ribosomal precursor RNA, to remain hydrogen bonded to 28s RNA and to have a role in maintaining the configuration of the 28s RNA.

Evidence of modification in the in vivo tRNA population has

been reported in cells undergoing differentiation or a similar process and also in certain virus infected cells. Firstly, I propose to discuss the non-viral changes. These can be divided into two main classes - those in bacterial systems and those in higher organisms.

REGULATION AND DIFFERENTIATION

Bacterial Systems

In bacterial systems there is evidence that a few tRNA species or perhaps, more correctly, aminoacyl-tRNAs are involved in regulatory functions.

Evidence exists that the level of histidinyl-tRNA present in Salmonella typhimurium performs a regulatory function. In mutants which are deficient in loadable $tRNA^{his.}$ molecules or possess aberrant histidinyl transferases, the growth rate of the organism and the level of repression of the operon for the appropriate biosynthetic enzymes are limited by the supply of histidinyl-tRNA (Silbert, Fink & Ames, 1966; Roth & Ames, 1966).

In E. coli, alterations in the intracellular concentrations of isoleucyl and phenyalanyl-tRNA transferases (and therefore perhaps of the corresponding aminoacyl-tRNAs) are observed to

parallel changes in the composition of the growth medium (Nass & Neidhardt, 1967).

For example, removal of phenylalanine from the medium causes a diminution in the growth rate, a derepression of the appropriate biosynthetic enzymes and an increased activity of only the phenylalanyl-tRNA transferase (twice normal). Elevated levels of transferase activity are still detected after 10 hours in minimal medium, although the growth rate is, by then, increasing again due to the adaptation of the organism to its altered environment. External provision of phenylalanine leads to almost complete suppression of the phenylalanyl-tRNA transferase. In general, therefore, these particular transferases are not fully induced when the amino acid in question is present but are active during derepression of the corresponding biosynthetic enzymes.

The relative abundance of chromatographically separable phenylalanyl-tRNA species and isoleucyl-tRNA species are also correlatable with the growth conditions of E. coli (Wettstein, 1966). In minimal, non-aerated medium a form of phenylalanyl-tRNA (I) hardly detectable in bacteria maintained on well-aerated, enriched medium, is observed. A similar, abnormal phenylalanyl-tRNA has also been reported to accumulate in bacteria maintained in iron

deficient medium (Wettstein & Stent, 1968). The proportion and physical state of an isoleucyl-tRNA (III) is also different in exponentially growing, compared to leucine starved, E. coli cultures (Yegian & Stent, 1969a) and it has been suggested that the differences observed may be correlatable with the oxidation-reduction state of the tRNAs themselves.

B. subtilis, when in favourable medium, passes through a period of exponential growth, a period of sporulation and a stationary phase and it has been shown that the compositions of the $tRNA^{lys.}$ (Lazzarini, 1966) and the $tRNA^{val.}$ populations are associated with this cycle (Heyman, Seror, Desseaux & Legault-Demare, 1967). B. subtilis contains 2 species of $tRNA^{val.}$ which are chromatographically distinct - I and II. During the phase of exponential growth, the ratio between valyl-tRNA I and valyl-tRNA II on MAK is 1.4, while in sporulating cells the ratio is 0.6 (Doi & Kaneko, 1966). Explanations such as aggregation, partial degradation and changes in molecular configuration have been eliminated. The alteration is generally considered attributable to an increased concentration of valyl-tRNA I rather than to a decreased concentration of valyl-tRNA II. More detailed investigation has revealed that the change occurs either at the end

of log phase or at the beginning of sporulation and that the value may have returned to normal late in the sporulation period. The alteration in the tRNA population has not been directly correlated with sporulation since a mutant of B. subtilis which cannot sporulate has an almost normal wild type pattern (Doi, Kaneko & Igarashi, 1968). It is, therefore simplest to postulate that the new distribution of valyl-tRNAs is due to changes in the components of the growth medium induced by sporulation as distinct from sporulation itself. This explanation is rendered more plausible by the report that the chromatographic distribution pattern of seryl-tRNAs is medium dependent (Doi, Kaneko & Goehler, 1966). It does not, however, appear to be the complete answer to the situation for valyl-tRNAs since an alteration of similar magnitude is not observed following step-up or step-down culture (Doi, Kaneko & Igarashi, 1968).

The pattern of $tRNA^{lys.}$ is also found to be altered at various stages in the growth cycle (Lazzarini, 1966). In particular, tRNA isolated from B. subtilis late in the sporulation cycle, i.e. when cell walls are forming, contains a peak of lysyl-tRNA, not observed on MAK in preparations from log phase cells. The appearance of the new lysyl-tRNA may be correlated with an increased

receptor activity for lysine. A subsequent report, however, suggests that the alteration is medium dependent and not due to sporulation per se (Lazzarini & Santangels, 1967).

To explain the variation at different stages in the B. subtilis growth cycle or the medium dependence of the tRNA populations, it can be postulated that (a) differential transcription occurs or (b) the efficiency of aminoacylation changes, due to alterations either of the tRNA structure or of the activity or specificity of the aminoacyl-tRNA transferases. The function of the alteration in tRNA balance has not been elucidated although, it has been suggested that, since the N-terminal amino acid of the main B. subtilis protein is serine, the extra seryl-tRNA peak may be of a special type required for a rapid rate of protein synthesis (Doi, Kaneko & Goehler, 1966). Alternatively, if the multiple aminoacyl-tRNA peaks are specifically related to the degeneracy of the code, it may be that the observed alterations mirror differences in the frequency of codons under translation.

Higher Organisms

Iso-accepting aminoacyl-tRNAs occur with greater frequency in cells of higher organisms. This may, in fact be attributable to cellular compartmentation (Buck & Nass, 1968; Barnett & Brown,

1967) but may also provide a clue to cellular regulation and differentiation.

In mammalian systems, it has been shown that, in most cases, there is no qualitative alteration in the chromatographic distribution patterns of the majority of aminoacyl-tRNAs, either at tissue level or between different species. There are, however, reports of several important exceptions. In particular,

(1) A form of histidinyl-tRNA not observed in other calf tissues is present in tRNAs derived from liver (Wevers, Baguley & Ralph, 1966).

(2) In mouse plasma tumour cells producing different γ globulins, the balance between different forms of seryl-tRNA, as resolved by Reverse phase chromatography, is altered (Yang & Novelli, 1968). In cells producing IgG molecules, 27% of the total seryl-tRNA is present in a form (type I) which is not detectable in cells of the 'IgA type'. 30% of the seryl-tRNA in IgA producing cells, on the other hand, exists in another form (type IV). Type IV is not detected in tRNA from 'IgG type' cells.

(3) The tyrosyl-tRNA of fibroblastic cells (except human derived ones) differs in MAK elution characteristics from

that of epithelial cells and may represent a more primitive type of tRNA (Holland, Taylor & Buck, 1967).

(4) Several aminoacyl-tRNAs derived from tumour cells differ from those observed in the corresponding normal cells. For instance, tyrosyl-tRNA from several tumour types shows altered MAK elution characteristics, as summarised in Table 2. Species of glycyl and seryl-tRNAs, undetected in normal mouse cells, are observed in tRNA derived from Ehrlich Ascites or Mouse Sarcoma cells (Taylor et al, 1967). Also, a species of lysyl-tRNA normal to rat liver cells is not produced in parallel preparations from livers of rats exposed to the carcinogen ethionine. It should be noted that this depression is not a characteristic of the neoplasms eventually induced (Axel, Weinstein & Farber, 1967).

It is also known, that the content of methylated bases in tRNA from tumorigenic sources differs from that of normal cells. Extensive methylation of the bases of tRNA has been observed in adenocarcinomas (Kaye, Fridlender & Salomon, 1966), mammary tumours (Bergquist & Matthews, 1962), human tumours and leukaemic leucocytes (Tsutsui, Srinivasan & Borek, 1966).

Table 2.

Cell Line	Tyrosyl-tRNA type
HeLa Cells	Equally epithelial and fibroblastic
Ehrlich Ascites Cells	Equally epithelial and fibroblastic
Adeno 7 transformed Hamster Cells	Equally epithelial and fibroblastic
SV40 transformed Hamster Cells	Fibroblastic predominate
Human Lymphatic Lung Cells	Epithelial predominate
Rous transformed Hamster Cells	Fibroblastic predominate

Comparison of the Composition of the Tyrosyl-tRNA Population from Different Tumorigenic Sources.

Tyrosine-tRNA was extracted and analysed as described by Taylor, Buck, Granger & Holland (1968) and is classified as epithelial or fibroblastic by its elution characteristics on MAK columns.

In mouse tumours (Hancock, 1967), there is, on average, a 2-10 fold elevation over normal in the level and rate of methylation, while in the Novikoff hepatoma the normal balance among individual methylases is upset (Tsutsui, Srinivasan & Borek, 1966). Alterations in the methylase specificity balance have also been observed following ethionine or SV40 virus (but not polyoma virus) induced neoplastic transformation (Hancock, 1968; Mittelman, Hall, Yohn & Grace, 1967). The tumour enzymes add further methyl groups to normal tRNA, fully methylated by homologous enzymes, and, in general, introduce unusual methylated bases. The structures of these bases are, in certain cases, as yet undetermined. Stimulation of methylation similar to that observed in tumour cells has been observed in preparations from foetal sources (Kaye & Leboy, 1968) but is not manifested in regenerating rat liver (Rodeh, Feldman & Littauer, 1967).

Alterations in tRNA populations and tRNA modifying enzymes have also been reported in plant and invertebrate systems undergoing differentiation.

(1) In wheat seedlings, species of seryl-tRNA, prolyl-tRNA and lysyl-tRNA, not present in the embryonic tRNA

populations, have been described (Vold & Sypherd, 1968).

(2) Early in embryogenesis of the sea urchin, a lysyl-tRNA, located only in the cytoplasm of unfertilised eggs, appears to migrate into and remain associated with the particulate fraction of the cell. Seryl-tRNA also appears to show a specific change (Yang & Comb, 1968).

(3) In the species Tenebrio molitor, which metamorphoses in 7 days from larva to beetle, cyclical changes occur both in the level of incorporation of methyl groups and in the specific activity of the methylases (Baliga, Srinivasan & Borek, 1965). Methylase changes are also demonstrable in tadpoles treated with thyroxine (Borek, 1968).

At present, it is not known whether the alterations observed in the tRNA populations are attributable to selective transcription of tRNA, to an altered complement of aminoacyl-tRNA transferases or to modification of existing species of tRNA. The last mentioned possibility - modification - is very real in view of the new methylation characteristics described in tumour cells and in systems undergoing differentiation.

VIRUS INFECTION

Alterations of the tRNA population have been reported following viral infection both with bacterial and animal viruses. These changes and others involving alterations of tRNA related enzymes, low molecular weight RNAs and ribosomes after virus infection are now presented. The categories are as follows:-

1. Effect on ribosomal function
2. Effect on low molecular weight RNAs
3. Effect on system for methylating tRNAs
4. Effect on aminoacyl-tRNA transferases
5. Effect on tRNA populations

1. Effect on Ribosomal Function

Virus infection has been shown in certain cases to cause alterations in functional ribosome units. For instance, the process of infection with herpes simplex virus has been correlated with polysome breakdown (Sydiskis & Roizman, 1967), while interferon mediated translational control has been attributed to configurational alterations of the ribosome (Marcus & Salb, 1966).

2. Effect on Low Molecular Weight RNAs

In at least two cases, additional species of low molecular weight RNAs have been observed following virus infection. The components, reported to be synthesised de novo, in KB cells infected for 10-14 hours with adeno 1 or adeno 2 virus are termed VA RNAs. (Rose, Reich & Weissman, 1965; Reich, Forget, Weissman & Rose, 1966). In comparison to the 5s like RNA species previously discussed (p. 28), VA RNA is located in the cytoplasmic and not the ribosomal fraction of the cell. Although similar to cellular 5s in respect to molecular volume, absence of methylated bases and pseudouridine, and in its inability to accept amino acids, it appears to differ in its rate of synthesis and base sequence (Forget & Weissman, 1967a). Neither the biological function of this RNA species, which is present at a concentration 10 times that of normal 'ribosomal' 5s, nor the identity of the specifying DNA (viral or cellular) have, as yet, been determined.

The low molecular weight RNA reported in T_4 infected E. coli (Baguley, Bergquist & Ralph, 1967) is attached to, or an integral part of, ribosomes. Its base composition resembles T_4 DNA but it is not the product of random degradation of high

molecular weight T_4 specified RNA. In this case also, no function of such an RNA species has been postulated.

3. Effect on tRNA Methylating System

(a) In T_3 infected E. coli (6-8 minutes post infection), methylation of both DNA and RNA is severely inhibited due to the presence of an enzyme which catalyses the breakdown of S adenosyl methionine - a required intermediate in methylation reactions (Gefter, Hausmann, Gold & Hurwitz, 1966). Bacteria, infected with other T phages or λ, do not contain such an enzyme. The enzyme, does not appear to play a major part in the T_3 lytic cycle since T_3 sam⁻, a mutant of T_3 incapable of producing functional enzyme molecules, is capable of normal lytic growth (Hausmann, 1967). No biological function can be postulated.

Methylation also appears to be affected in a more direct way in other viral systems.

(b) In 4s RNA from T_4 infected E. coli, the structure of the bases which are labelled with methyl groups from methyl methionine (Boezi, Armstrong & De Backer, 1967), differ from those in non-infected controls. In particular, the RNAs appear to contain a far higher proportion of

ribosylthymine. These bases may be correlatable with the unidentified methylated bases reported in T_4 specified sRNA by Daniel, Sarid & Littauer (1968).

(c) By far the best documented cases of large scale viral interference with methylation is that concerning induction in E. coli carrying λ by either heat or U. V. irradiation (Wainfan, Srinivasan & Borek, 1965).

When U. V. is used for induction there is an early diminution in the level of tRNA methylase activity in the E. coli extracts. This reduction is a sequel only to induction of lysogenic organisms and is due to the production of a stable dializable inhibitor which is not a direct product of U. V. irradiation, but the result of a metabolic process (Wainfan, Srinivasan & Borek, 1966). The methylase activity after this initial drop rises back to a level similar to the initial value. This activity differs from that in the uninduced host cell in that the enzymes which methylate uracil and adenine have activities reduced with respect to that of guanine methylase. It is not clear, whether the restoration of enzyme activity is achieved by the removal of the effect of the inhibitor or is the result of new synthesis of

enzymes.

On induction by heat a similar pattern is observed (Wainfan, 1968). However, the repression of the methylase activity of the uninduced host cell here can be shown to continue throughout the induction period. It is not dependent on protein synthesis. The subsequent rise in methylase activity is only observed if protein synthesis is permitted. The new activity appears, therefore, to be due to the formation of new heat stable methylases. Such results are in accordance with the induction of a new guanine methylase in SV40 transformed hamster cells (Mittelman et al, 1967).

(d) An increase of methylating enzyme activity or the appearance of tRNA containing an altered methylation pattern need not always be associated with virus infection. In foot and mouth disease virus (Vande-Woude, Arlinghaus & Polatnik, 1967) infection, diminished methyl incorporation into 4s is observed but this may merely reflect the overall decrease in tRNA synthesis.

4. Effect on Aminoacyl-tRNA Transferases

From the discussion on p. 19 it is obvious that aminoacyl-tRNA

transferases from genetically unrelated sources may differ in certain general characteristics and may be separable by chromatographic methods. If the bacteria and bacteriophages are sufficiently distant from an evolutionary standpoint, it is possible that alterations in the properties of one or more transferases may follow phage infection. To facilitate the investigation of this possibility, use was made of E. coli mutants each of which possessed an aminoacyl-tRNA transferase aberrant as regards its K_m value or in its response to amino acid analogues or its sensitivity to elevated temperatures. (Earhart & Neidhardt, 1967). To date, it has been reported that, following T_4 infection of these E. coli mutants, the properties of glycyl, histidinyl and phenylalanyl-tRNA transferases remain aberrant. The chromatographic characteristics of the phenylalanyl-tRNA transferase remain unaltered and all the enzymic activities are neutralised by antibodies against the bacterial enzymes. This suggests that the formation of aminoacyl-tRNA, in these cases, is mediated by unaltered pre-existing host enzymes.

Evidence obtained using T_4 infection of either wild type E. coli or a temperature sensitive mutant of that bacterium

inferred the appearance after infection of a different valyl-tRNA transferase (Neidhardt & Earhart, 1966). In the temperature sensitive system, the new activity appeared 2 minutes post infection and increased to a steady maximum (10% of that in the wild type) at approximately 10 minutes post infection, unless U. V. inactivated phage particles were used as inoculum when the activity increased linearly up to 25 minutes post infection. The new transferase not only displayed higher stability at 40° in vivo, loading 80% of $tRNA^{val.}$, but also survived extraction and was active in vitro at 37°. De novo protein synthesis was required after phage infection for its expression. Virus induced valyl-tRNA transferase was also detected subsequent to T_4 infection, or more generally T_{even} infection in wild type E. coli. The new enzyme, which differed chromatographically from the pre-existing enzyme, had a molecular weight twice that of the normal cellular transferase and comprised over 50% of the valyl-tRNA transferase activity in the infected cell. It was induced by mutants of T_4 known to be deficient in late functions. Initially, therefore, it seemed that phage T_4 coded for a valyl-tRNA transferase with properties different from those of the host bacterium. The enzyme was

classed as an early viral function.

However, the appearance of the 'new' enzyme was concomitant with the disappearance of the normal transferase, thus suggesting the 'new' transferase to be a modified version of the host enzyme. Experimental proof of conversion of a protein formed before infection was obtained by 'heavy isotope' labelling and immunological techniques (Chrispeels, Boyd, Williams & Neidhardt, 1968). The modifying factor appears to be a product of protein synthesis which perhaps leads to the aggregation of 2 molecules of E. coli valyl-tRNA transferase. A function for this transferase at the molecular level is, so far, undetected, MAK column chromatography having shown no difference in the valyl-tRNA population pre and post infection. The biological significance has not yet been elucidated either, but the kinetics of appearance of the modified enzyme after infection with wild type T_4, U. V. treated T_4 or genetically defective T_4 suggest a similarity to those of early enzymes. The enzyme modification may, therefore, be involved in the regulation of early versus late functions. The phenomenon is shown only with regards to valyl-tRNA transferase and by the related T_{even} phages. It is not demonstrable in T_5 or λ infections.

5. Effect on tRNA Populations

The most extensively studied system is infection of E. coli with T_{even} bacteriophage. Evidence in 2 systems has been obtained that the normal programme of sRNA synthesis and, therefore, probably the overall composition of the tRNA population, is altered following infection of E. coli with T_{even} bacteriophage. Both systems involve chromatographic examination of low molecular weight RNA radioactively labelled in vivo.

Distinct alterations were observed in the chromatographic behaviour on MAK columns of S^{35} labelled RNA extracted 5 minutes after infection with T_2 or T_4 bacteriophage (Hsu, Foft & Weiss, 1967). In particular, the characteristic distribution of the sulphur label into 3 peaks (1, 2 and 3), 2 of which (1 and 2) were predominant, was altered to a pattern which indicated a diminished rate of sulphur incorporation into peaks 1 and 2 and an increased rate of labelling of peak 3. Such changes were not observed following infection of E. coli with T_7, ØX 174 or MS2. Associated with the altered profiles was a change in the kinetics of the thiolation reaction.

Tillack & Smith (1968) have demonstrated an increased

incorporation of labelled guanine into tRNA after T_2 infection and have associated it with an alteration of the chromatographic elution pattern from Reverse phase (type II) columns. sRNA from non-infected E. coli was resolved into 3 radioactively labelled peaks (A, B and C) while similar material, derived 5 minutes after T_2 infection, contained no labelled material in position A, had a decreased concentration of label in position C and exhibited 2 peaks of radioactivity in position B.

Assuming that the differences in the 2 systems outlined are consequences of T_{even} infection, they are explainable in terms of one of the following mechanisms.

(a) Synthesis of virus coded tRNA

(b) Viral modifications of host coded tRNA

(c) Viral control of host cell tRNA transcription (leading to the production of a limited number of tRNA species either by inhibiting the formation of certain species or stimulating the synthesis of others).

The experiments of Tillack & Smith (1968) on guanine incorporation have not, as yet, been taken far enough to elucidate the mechanism of viral interference. However, the

system of Hsu, Foft & Weiss (1967) has been investigated further and here it was found that the tRNA molecules localised in peak 3 in infected preparations did not contain a significantly high percentage of thionucleotides than did species present in the uninfected bacterium. This renders unlikely any explanation of the increased sulphur labelling in terms of modification of pre-existing 'peak 3' tRNAs, by the introduction of extra thionucleotides. Since it also appears that the new tRNAs located in peak 3 are not derivatives of those found before infection in positions 1 or 2, the experimental data suggest that the observed transition from the normal S^{35} labelling pattern to the infected tRNA profile is not the result of modification of pre-existing host species. Instead, the changes are attributable to de novo synthesis of host or virus coded tRNAs.

The feasibility of virus induction of tRNAs was reinforced by the experiments of Smith et al. (Smith, Abelson, Clark, Goodman & Brenner, 1966). Their results demonstrated that after infection with a defective transducing phage 80 which carried the structural gene of suppressor III tyrosyl-tRNA, a new $tRNA^{tyr.}$

could be observed in the bacteria. No alteration in the overall concentration of the tyrosyl-tRNA was noted during a typical lytic cycle and certain conditions had to be fulfilled before the amount of this tRNA reached a detectable level. Namely, an artificially prolonged growth cycle was necessary and so was a certain degree of protein synthesis early in infection. This system shows that in bacterial cells the machinery exists for the transcription of tRNA genes present in viral genomes and also that the tRNA product can be introduced into the protein synthesising apparatus of the cell. The results also contain a warning against over-emphasising quantitative observations.

The foregoing discussion has assumed that low molecular weight RNA may be equated with tRNA and, although in the case involving sulphur incorporation this may be a valid assumption, direct investigation of aminoacyl-tRNA systems is warranted. Such data, obtained either by MAK column chromatography or Reverse phase chromatography (type I), has been reported by the Sueoka group (Sueoka & Kano-Sueoka, 1964; Kano-Sueoka & Sueoka, 1966; Sueoka, Kano-Sueoka & Gartland, 1966) and by Waters and Novelli (1967), respectively.

The elution profile of 17 aminoacyl-tRNAs from T_2 infected and uninfected E. coli were investigated by chromatography on MAK of doubly labelled mixed preparations. In fourteen of the cases, no differences were observed between preparations before and 8 minutes after infection. Slight ambiguity was detected with isoleucyl and seryl-tRNAs, but a significant difference was only observed in the case of leucyl-tRNA. This alteration was not attributable to a change in leucyl-tRNA transferase but to the fact that the complement of $tRNA^{leu.}$ in T_2 infected cells differed from that in the uninfected cell. Normal leucyl-tRNA eluted from MAK as 2 major peaks, with peak I predominating, while the exact profile of leucyl-tRNA from infected cells, although at no time after infection coincident with the normal pattern, was dependent on the extent to which infection had proceeded. Very early in infection (1 minute post infection) for example, a new small peak eluting before peak I was observed. This peak, termed leucyl-tRNA F, was transient, the precise half-life being determined by the state of the infecting phage. By 8 minutes post infection, it had generally disappeared and, at this stage, viral alteration was manifested as a decrease of peak I

material, with a concomitant increase in peak II material. The peak II material, which initially contained a type of $tRNA^{leu.}$ unable to accept leucine in a heterologous reaction, now appeared to be composed of two types of tRNA. One of these, termed leucyl-tRNA R, was an active acceptor during heterologous loading experiments.

Similar modifications of leucyl-tRNAs were found after infection of E. coli mutants or Shigella 60 with T_2 phage or other T_{even} phages (T_4 and T_6) but not following T_{odd} bacteriophage infection or after induction of λ.

The coding properties of leucyl-tRNAs derived from uninfected and T_2 infected E. coli have been examined by polynucleotide stimulating binding to ribosomes of different RNA fractions from MAK. It appears that peak I $tRNA^{leu.}$ normally binds with CUG and poly UC whereas peak II binds poly (UC) and to a lesser extent poly (U) or poly (UG) and the trailing edge binds UUG codons in poly (UG). Peak F material, isolated at 1 minute post infection, binds poly (UG) but none of the component triplets of this polynucleotide. At 8 minutes post infection, it would appear that peak II material binds an increased amount of CUU, CUC and poly (U)

(Kano-Sueoka, Nirenberg & Sueoka, 1968).

In the light of the experimental observations summarised above, Kano-Sueoka and Sueoka (1966) postulated that the alterations detected in the elution pattern of leucyl-tRNA on MAK columns were attributable to phage induced enzymic modification of a pre-existing species of host $tRNA^{leu.}$ (component I to be exact). Their reasoning was as follows -

1. DNA from T_{even} phages, as distinct from the genetically distinct T_{odd} phages (Schildkraut, Wierzchowski, Marmur, Green & Doty, 1962), was required for the expression of leucyl-tRNA alterations. There was no dependence of viral expression on the species of the host. This was, therefore, a phage specific reaction.

2. Protein synthesis was necessary, thus suggesting an enzymic nature for the alteration.

3. Leucyl-tRNA transferase was normal after infection and so the $tRNA^{leu.}$ population itself must have been the site of alteration.

4. The relative amounts of leucyl-tRNA to other aminoacyl-tRNAs remained unaltered after infection and

therefore no de novo synthesis had occurred.

5. The new leucyl-tRNA components possessed a characteristic in common with bacterial leucyl-tRNA I in that they were heterologously loaded with yeast leucyl-tRNA transferase.

No conclusion as to the nature of the modification or to its biological significance was reached although, based on data obtained from polynucleotide binding, it was suggested that E. coli $tRNA^{leu.}$ is transcribed from two different cistrons (A & B), (Nirenberg, Caskey, Marshall, Brimacombe, Kellogg, Doctor, Hatfield, Levin, Rottman, Pestka, Wilcox & Anderson, 1966). All species of tRNA observed in host or T_2 infected cells were postulated to be different modifications or stages in modification of one of the two basic designs.

However, no DNA-sRNA hybridisation studies have been undertaken by Sueoka and the results, when intepreted as follows, may be equally well explained by the formation of virus coded $tRNA^{leu.}$ species.

1. The production of virus coded tRNA species would also require viral DNA and be independent of the identity

of the host cell. There is also no reason to suppose that it would be possible to distinguish between species of tRNA coded by 2 members of the T_{even} group of phages but not between modified versions of host coded species produced by these phages.

2. Protein synthesis might be required to permit expression of the virus gene in question, especially since chloramphenicol is known to prevent the formation of detectable amounts of virus coded tRNA in other systems.

3. A virus coded tRNA could be loaded by bacterial transferases as easily as a virus modified host species.

4. Failure to detect appreciable changes in total quantity and relative amount of leucyl-tRNA post infection would not necessarily preclude de novo synthesis of $tRNA^{leu}$. (due to experimental detection limitations).

5. Virus coded tRNA species might be loadable by yeast enzyme especially since the structural basis for amino acid attachment by heterologous transferases is not well defined.

Indeed, at present, the latter interpretations are more easily correlated with the DNA-sRNA hybridisation data of Weiss, Hsu, Foft & Sherberg (1968) which are discussed on p.59.

Waters and Novelli (1967) have studied the same host virus system using a technique (Reverse phase chromatography type I) of greater resolving power than MAK. Only the leucyl-tRNA population was investigated. Under the chromatographic conditions used, leucyl-tRNA from uninfected E. coli was resolved into 3 and only 3 components (I, II & III) with peak I predominating over peaks II and III. By 6 minutes after phage infection, no qualitative change in the eluted species had occurred but quantitative changes between peaks I, II and III were observed. Relative to peak I, peaks II and III increased in the tRNA from infected cells over that seen in the normal case. The altered distribution pattern probably corresponds to the changes in leucyl-tRNA population detected at 8 minutes post infection with MAK chromatography. Such changes in the relative proportions of leucyl-tRNA components were maintained until 2 hours post infection, i. e., until late in the viral growth cycle. A further alteration in the overall composition of leucyl-tRNAs present at

2 hours post infection, was also observed. At least one and probably 2 new peaks (IV & V) had appeared. These peaks, first detectable at 32 minutes post infection, eluted after peak III and were never observed in tRNA preparations from uninfected E. coli. Further data obtained suggested that the changes observed following T_2 infection were phage specific and not merely a consequence of under methylation or altered growth conditions in the bacterial cultures. Waters & Novelli (1967) concluded that the change observed early in infection was due to modification as postulated by Sueoka, Kano-Sueoka & Gartland (1966) and remained uncommitted as to the nature of peaks IV and V, since insufficient experimental data was available. However, subsequently, they have suggested that early in T_2 infection the major species of $tRNA^{leu.}$ is not modified to another form still capable of accepting leucine but is instead completely inactivated (Waters & Novelli, 1968).

Alterations in the composition of the sRNA fraction subsequent to T_4 infection have recently been investigated by Weiss et al, (1968) and Daniel, Sarid & Littauer (1968) using DNA-sRNA hybridisation techniques.

Daniel, Sarid & Littauer (1968) have shown that sRNA

derived from T_4 infected E. coli contained a type of sRNA not observed in the uninfected cell. This RNA was characterised by possessing sequence homology with 0.05-0.2% of the T_4 genome and with less than 0.001% of the E. coli DNA. Further, the base composition of the RNA recovered from DNA-RNA hybrid molecules did not resemble T_4 mRNA and contained significant amounts of pseudouridine and traces of 2 other methylated bases. Since such modified bases are normally located only in tRNA and since the hybridisation data seem qualitatively sound, there is a strong possibility that T_4 codes for one or more tRNAs. The data do not, however, provide a rigorous proof.

In contrast, the hybridisation data, reported by Weiss et al, (1968), in the same host virus system, are more extensive and their interpretation is consequently more clearcut. Either sRNA pulse-labelled with $[S^{35}]$ or tRNA loaded in vitro with labelled amino acids were used for the hybridisation experiments.

The results obtained using highly labelled S^{35} RNA from T_4 infected E. coli and DNA from T_2, T_4 bacteriophages and E. coli indicated that the labelled RNA was effectively bound to T_4 and T_2 DNA but that the degree of attachment to E. coli or T_7 DNA was

insignificant. Since neither T_4 mRNA nor E. coli tRNA competed effectively with this $[S^{35}]$ tRNA for its specific attachment site on T_4 DNA and since T_4 mRNA has been shown to lack thionucleotide bases, it can be concluded with certainty that tRNA homologous to T_4 DNA is synthesised early in infection with this phage. Similar experiments revealed that 30% of peak 3 material from MAK columns i. e. the fraction previously shown to be increased in 'infected' tRNA preparations - was hybridisable to T_4 DNA.

Direct determination of the identity of the virus specified aminoacyl-tRNAs was investigated by a technique designed to detect annealing of DNA and homologous aminoacyl-tRNA, labelled in the amino acid moeity. Specific hybridisation of radioactivity derived from $[^3H]$ leucyl-tRNA from T_4 infected E. coli to T_4 DNA was demonstrated. Exposure to pH conditions high enough to break aminoacyl bonds, but not to disrupt DNA-RNA hybrids, (1M-Tris-HCl buffer pH 9.0), resulted in a loss of the radioactivity previously associated with the hybrid structure. Such data demonstrated conclusively that at least part of the leucyl-tRNA population present in T_4 infected E. coli was specified by the viral genome. Further,

of the 3 types of leucyl-tRNA, separable by MAK chromatography of infected tRNA, peak I was comprised of host tRNA and peak 3 of T_4 specified material. Therefore, although these MAK column chromatographic patterns are not exactly correlatable to those described by Kano-Sueoka & Sueoka (1966), it seems reasonable to deduce that the alterations detected in the leucyl-tRNA population early in T_{even} infection by these workers and by Waters & Novelli (1967) are due to synthesis of at least one species of virus coded $tRNA^{leu.}$. (The possibility of concomitant virus modification is not experimentally excluded). The hybridisation data give no indication of the nature of the 2 'species' of $tRNA^{leu.}$ described late in infection. Hybridisation between T_4 DNA and "infected" prolyl-tRNA was also detected. The T_4 genome therefore codes for at least one leucyl-tRNA species and perhaps also for prolyl-tRNA.

An additional change in the population of E. coli tRNAs is also reported following T_4 infection. In particular, suppression of the chain terminating codon UAA by Su^{4+} was abolished, thus suggesting that minor tRNA host species may be modified as a result of T_4 infection (Brenner, Kaplan & Stretton, 1966).

Other bacterial host-virus systems, namely R_{17}-E. coli, and Qβ-E. coli have also been examined (Hudson & Paranchych, 1968; Hung, 1966). Briefly, the data reported appear to indicate that R_{17} does not specify tRNA and that the response of prolyl-tRNA derived from Qβ infected cells to poly C in the ribosome binding technique differs from that from non-infected E. coli.

The tRNA population of BHK21/13 cells is altered as a consequence of infection with the ∝ strain of herpes simplex virus (Subak-Sharpe & Hay, 1965). By demonstrating that purified sRNA derived from virus infected cells hybridised to the viral DNA while that from uninfected cells did not, it was possible to conclude that herpes virus may code for new sRNAs with many of the characteristics of tRNAs. Quantitation of the data showed that the virus may code for, at most, 19 sRNA molecules. Both the qualitative and quantitative conclusions drawn are, of course, subject to the experimental limitations mentioned on page 154 with regard to hybridisation data. The detection of virus specified tRNA species was associated with the appearance in virus infected cells of a "new" arginyl-tRNA species, as detected by MAK column chromatography of differentially

labelled preparations (Subak-Sharpe, Shepherd & Hay, 1966). Part of the radioactivity derived from the infected arginyl-tRNA eluted at a higher ionic strength than did that derived from the non-infected control. The simplest explanation of these results is that the herpes virus genome codes for characteristic tRNA species, at least one of which specifically attaches arginine.

In the foregoing discussion, viral interference has been demonstrated to influence many of the components of the protein synthetic mechanism - either by introducing new virus coded species of tRNA, by specifying modifications of pre-existing tRNAs, by affecting at least one aminoacyl-tRNA transferase or by altering ribosomal components and disturbing their function.

The biological significance of these differences, observed in a variety of systems, has not yet been elucidated. In certain cases, even the normal role of a component, far less that of its viral counterpart is known, e.g., 5s and VA RNA. It is, however, possible to assume that some of the alterations discussed eventually operate by effecting the introduction of (either by modification or de novo synthesis) one or more specific tRNAs and that the tRNA population, so produced, may differ from that present prior to infection in the relative

proportions of iso-accepting tRNAs and/or in its capacity to recognise certain codewords. Such aminoacyl-tRNA may, of course, not be involved in protein synthesis or it may be envisaged that they are concerned either directly or indirectly in the translation mechanism, i.e. they may be used as necessary adaptors or may be blocking initiation for example. By correlating the functions altered, and the natures of the systems involved, it seems probable, at least in certain cases, that the molecules may be serving as adaptors for those codons in RNA which, under normal circumstances, either do not occur or are infrequently encountered.

Inter-relationships of mRNA composition and tRNA populations have been shown to occur in seryl-tRNA populations from different immunoglobulin producing tumours (Yang & Novelli, 1968) and in a mutant of B. paracoli with an elevated proportion of G+C bases (Gause & Grünberger, 1968). Such a situation could be theoretically envisaged to occur in herpes simplex infection of BHK21/13 cells. The rationale as postulated by Subak-Sharpe is explained more fully as follows.

The DNA of herpes simplex virus has a base composition of 68% G+C whereas mammalian cells contain DNA with a base

composition of 40-44% G+C. Comparison of nearest neighbour base sequence analyses of these two DNAs show more fundamental differences - discrepancies in the frequency of occurrence of the doublets CpC, GpG, GpC and CpG. Indeed the doublet CpG is almost entirely absent in cellular DNA but frequently occurs in the viral DNA. This situation will be mirrored in the viral mRNA, if it is assumed that most of the viral DNA is polypeptide specifying, i. e., viral mRNA will contain a much higher proportion of these doublets than does the host mRNA. Since, as a consequence of natural selection, the tRNA population of the host cell should be optimally adapted to the translation of cellular mRNA, it would seem reasonable to suggest that unless extensive redundancy exists within this population, tRNAs recognising the codons (1) CGX (X = U, A, C or G), (2) UCG, CCG, ACG, GCG, and also (3) (GCU, GCC, GCA) (CCU, CCC, CCA) and GGX would be poorly represented. On the basis of the Wobble hypothesis, the shortage would be most acute where the doublets in question occupy the first two positions of any codon $\overset{1\ 2\ 3}{(CGX)}$. Assuming that the genetic code is universal, the codons detailed above are assigned to (1) arginine, (2) serine, proline, threonine, alanine and (3) alanine, proline and glycine

respectively. Consequently, to facilitate rapid translation of viral mRNA, the virion would have to alter the balance of the tRNA population either qualitatively or quantitatively, with respect to these aminoacyl-tRNAs. No alteration with respect to lysyl-tRNA, say, (recognising AAA or AAG) would be predicted. In other words, the virus would either have to (i) code directly for new tRNA molecules of the codon recognising class required, (ii) alter the codon recognition of pre-existing host tRNAs, or (iii) selectively induce the production of these species of host tRNAs.

It follows, therefore, from these theoretical considerations that any mammalian virus with DNA of a high G+C content should experience severe translational problems during multiplication. Pseudorabies virus - a large icosohedoral virus of the Herpes virus group - contains double stranded DNA with a G+C content of 72% (Ben-Porat & Kaplan, 1962) and is, therefore, eminently suitable to provide a further test of the hypothesis outlined. In addition, this virus, which multiplies in the cell nucleus, but directs polypeptide synthesis in the cell cytoplasm (Fujiwara & Kaplan, 1967; Ben-Porat, Shimono & Kaplan, 1969), has many technical advantages over herpes simplex virus since it

has a much more rapid growth cycle, a higher burst size and shows early cytopathic effect (Kaplan & Vatter, 1959). The following investigations were designed to compare the low molecular weight RNA populations, in particular the tRNA population, in uninfected and pseudorabies virus infected cells.

MATERIALS

MATERIALS

a. Virus

A strain derived by 3 x plaque purification from a stock preparation of pseudorabies virus (Kaplan & Vatter, 1959) was used throughout. Herpes virus used was strain of HFEM (Russell, Gold, Keir, Omura, Watson & Wildy, 1964).

b. Tissue Culture Cells

BHK21/13 cells were a continuous line of hamster fibroblasts described by Macpherson & Stoker (1962).

c. Enzymes

Ribonuclease and deoxyribonuclease were obtained from the Sigma Chemical Co., St. Louis, Missouri or from Worthington Biochemical Corporation, Freehold, New Jersey.

T_1 RNase was obtained from either the Worthington Biochemical Corporation or from Sankyo & Co., Tokyo.

Pronase B grade was obtained from Calbiochem Ltd., London, W. 1.

d. Radiochemicals

All labelled compounds used were obtained from the Radiochemical Centre, Amersham.

e. Membrane Filters

Bact. T Flex Membrane filters were obtained from Schleicher & Schuell & Co., Keene, New Hampshire. HAWP 0.45 μ pore diameter membrane filters were obtained from the Millipore Corporation, U.S.A.

Glass Chromedia filters were obtained from Whatman.

f. 2, 5-diphenyloxazole (PPO), 1,4-bis-(2-(5-phenyl-oxazolyl)) - benzene (POPOP) and hyamine hydrochloride were obtained from Thorn Electronics Scintillator Division, Tolworth, Surrey.

g. Chromatographic Materials

Sephadex-G100 was supplied by Pharmacia Ltd., Uppsala, Sweden.

DEAE cellulose ion exchanger was obtained from Whatman.

High flo Supercel was obtained from Koch Light Laboratories, Ltd., Colnbrook, Bucks.

DMCS treated and washed Chromosorb W was obtained from Johns Manville Products Corporation.

Aliquot 336 (Methyl,tricaprylyl Ammonium Chloride) was obtained from General Mills, Kankee, Illinois.

Freon 214 (tetrachloro, tetrafluoropropane) was obtained from DuPont de Nemonts & Co., Wilmington, Delaware.

Methylated albumin was kindly gifted by Dr. J. Hay and was stored over KOH in a dessicator at 4°.

h. ATP, AMP and CTP were obtained from Calbiochem Ltd., London, W. 1., as was a complete set of 19 grade A amino acids.

All analar chemicals except CsCl were obtained from BDH Ltd., Poole, England. CsCl was obtained from Hopkin & Williams, Ltd., Essex.

Composition of media and standard solutions

Eagle's Medium

A modification of Eagle's medium (Busby, House & Macdonald, 1964) containing 100 units/ml. of Penicillin, 100 µg./ml. of Streptomycin, 0.002% phenol red and 0.2 µg./ml. of the antimycotic agent n-butyl, p-hydroxy benzoate was used.

Tryptose phosphate broth consisted of a 2.95% solution tryptose phosphate broth (Difco Bacto) in distilled water.

ETC growth medium was composed of 80% Eagle's medium, 10% Tryptose phosphate broth and 10% calf serum.

EC growth medium was composed of 89% Eagle's medium and 11% calf serum.

ESOH growth medium was composed of Eagle's medium supplemented with 50 mμ-moles serine/ml., 100 mμ-moles ornithine/ml. and 100 mμ-moles hypoxanthine/ml.

ETHu growth medium was composed of 80% Eagle's medium, 10% Tryptose phosphate broth and 10% human serum.

Agar Overlay Medium was prepared by mixing 2.5 ml. of calf serum and 25 ml. of 3.6% Difco Bacto Agar with 75 ml. of Eagle's medium (1.3 times normal concentration).

Phosphate buffered saline A (PBS A) was a solution of 0.17 M-NaCl, 3.4 mM-KCl, 10 mM-Na_2 HPO_4 and 2 mM-KH_2PO_4, pH 7.4 (Dulbecco & Vogt, 1954).

Tris-saline was a solution 25 mM Tris-HCl buffer pH 7.4, 0.14 M-NaCl, 5 mM-KCl, 0.7 mM-Na_2HPO_4, 5 mM-dextrose, containing 0.002% phenol red, 100 units/ml. of Penicillin and 100 μg./ml. of streptomycin.

Versene solution consisted of EDTA dissolved at a concentration of 0.6 mM in PBS A to which 0.002% of

phenol red had been added.

Trypsin-versene solution consisted of 1 volume of 0.25% trypsin solution (Difco trypsin in Tris-saline) in 4 volumes of 0.6 mM-versene, i.e. the final concentration of trypsin was 0.05%.

Standard Saline Citrate (SSC) was a solution of 0.15 M-NaCl and 15 mM-trisodium citrate, pH 7.0.

Acetic/ethanol mixture consisted of 3 parts glacial acetic acid and 1 part ethanol.

Chloroform/isoamyl alcohol mixture consisted of 50 parts chloroform and 1 part isoamyl alcohol.

90% Phenol was prepared by mixing 90 ml. freshly distilled phenol with 10 ml. of distilled water. The solution could be stored in a dark bottle in an atmosphere of nitrogen without discolouration occurring.

Toluene Scinstant for radioactive counting was prepared by dissolving 12.5 g. of PPO and 0.75 g. of POPOP in 2.5 l. of Analar toluene.

Bentonite

A 2.5% suspension of bentonite in 10 mM-sodium acetate

pH 6.0 was used throughout. The preparation was as follows. Bentonite powder was suspended at 50 mg./ml. in distilled water and the fraction sedimenting between 6,000 g and 20,000 g collected. This fraction, suspended in 0.1 M-EDTA at 10 mg./ml., was stirred mechanically for 24 hours at room temperature. The pellet produced by recentrifugation at 20,000 g for 15 minutes was suspended in and dialysed against distilled water. The fraction sedimenting at 20,000 g was recovered and suspended at 25 mg./ml. in 10 mM-sodium acetate, pH 6.0 (Cantoni & Davies, 1966).

CMC Medium was prepared by adding 50 ml. of Eagle's medium (1.3 x normal concentration) to an autoclaved suspension of (ether and alcohol) washed CMC (carboxymethyl cellulose) in distilled water (2 g./100 ml.). The medium was stored at 4°.

Kieselguhr was prepared by boiling suspension of Koch Light High flo Supercel (20 g. suspended in 100 ml. 0.1 M-NaCl, 50 mM-sodium phosphate buffer, pH 6.25) for a few minutes and allowing to cool.

MAK was prepared by mixing 5 ml. of a 1% solution of

methylated albumin (freshly dissolved) with 100 ml. of Kieselguhr suspension which had been prepared as previously described (Mandell & Hershey, 1960).

Pronase

Pronase was self digested by incubation for 2 hours at 37°.

Formol Saline (10%) consisted of 4% v/v formaldehyde in 85 mM-NaCl, 0.1 M-Na_2SO_4.

Giemsa Stain

A 1.5% suspension of Giemsa in glycerol, heated at 56° for 90-120 minutes and diluted with an equal volume of methanol was used as stock (Dacie, 1956).

METHODS

METHODS

BIOLOGICAL METHODS

CELL CULTURE SYSTEMS

Low passage BHK21/13 cells were seeded in 200 ml. of ETC medium or 180 ml. of EC medium into rotating 80 oz. Winchester bottles (10^7 cells per bottle), and incubated in an atmosphere of 5% CO_2 at 37° on a roller bottle culture rack of the type described by House and Wildy (1965). 2-3 days later, when a confluent monolayer, 4×10^8 cells/bottle, had formed, the cells were removed from the walls of the bottle by treatment with Trypsin-versene, washed in ETC or EC and dispensed into roller bottles at approximately 2×10^7 cells per bottle. The cell line was subsequently maintained by similar serial dilution. Roller bottles containing confluent monolayers, produced as already described, were used for most preparative procedures. Cell cultures were also grown in baby bottles, in 50 mm. plastic plates and in 50 mm. plastic plates containing 3 x 13 mm. glass coverslips. In the latter cases, the cultures were incubated in a constant temperature incubator flushed with a 5% CO_2 air mixture.

BHK21/13 cultures, in which metabolic function was depressed were produced by maintaining sparse cell monolayers for 4 days

in serum deficient medium (Bürk, 1967). ESOH was used for preexisting monolayers but with cell suspensions this medium was supplemented with 0.25% calf serum to facilitate cell adhesion.

Procedure for Infecting Cells with Pseudorabies Virus

(a) High Serum System

EC growth medium was removed from roller bottle cultures, containing approximately 4×10^8 BHK21/13 cells per bottle, and replaced by either a 20 ml. inoculum of pseudorabies virus (10^{10} plaque forming units per bottle in EC medium) or 20 ml. of EC medium. After a viral adsorption period of 60 minutes at 37°, the cultures were fed with a further 100 ml. of EC medium and incubated at 37° till harvesting.

(b) Serum Deficient System

ESOH medium, removed from roller bottle cultures in a sterile fashion, was maintained in an atmosphere of 5% CO_2 at 37°. The cultures were either mock-infected with 20 ml. of used medium or inoculated with pseudorabies virus in 20 ml. of the used medium at a multiplicity of exposure of 20 plaque forming units per cell. After a viral adsorption

period of 60 minutes at 37°, the cultures were fed with 100 ml. of the used medium and incubated at 37° till harvesting.

Production of Pseudorabies Virus Stocks

The Kaplan strain of pseudorabies virus, propagated in BHK21/13 cells formed the original stock (Kaplan & Vatter, 1959). The strain was genetically purified by three successive "single" plaque isolations. The appearance of one plaque in one culture in twenty was used as the criterion of "single", since this implied that the plaque had arisen from only one virus particle. At each stage, the plaques obtained were propagated in BHK21/13 cells for storage at -70° in Tris-saline Ca 10%.

BHK21/13 cell cultures in roller bottles, when newly confluent, were infected in 20 ml. ETC at a multiplicity of 1 plaque forming unit per 300 cells. The virus inoculum was allowed to adsorb to the cells for 1 hour at 37° as the bottle rotated. After adsorption 100 ml. of ETC medium was added and the bottle returned to the roller rack to be incubated at 37° for 27-36 hours. After this time, the cells were harvested by shaking the bottle to dislodge the cell sheet into the medium, the excess medium being removed by centrifugation for 10 minutes at 900 g. The virus containing

cell pellet was disrupted by sonication for 5 minutes at 60 m. amperes in the Dawe Soniclean Generator. After centrifugation at 900 g, the supernatant, which contained the virus particles, was removed to 4° while the debris was resuspended in Tris-saline Ca 10% (2.5 ml. for every roller bottle used initially), and then sonicated and centrifuged as before. The supernatant fraction was again removed and pooled with that obtained at the previous step. This combined fraction was subsequently stored at -70° for use as viral inoculum, or for extraction of the viral DNA.

Plaque Assays for Pseudorabies Virus

(a) Dilutions of virus in Tris-saline Ca 10% were added to suspensions of BHK21/13 cells at 4×10^6 cells per ml.. The infected cells were maintained in suspension for 1 hour at 37° to allow adsorption to occur and then aliquots of 4×10^6 cells were dispensed, in 4 ml. of ETC, into 50 mm. plastic plates. After incubation at 37° for 3 hours, the medium was removed and replaced by 3 ml. of agar overlay medium. The plates were then incubated at 37° and 27 hours later the cultures were fixed by exposure to formol saline for 10 minutes. The agar overlay was then floated off in warm

water and the cell sheet stained with Giemsa for 10 minutes at room temperature. A maximum of 200 plaques per plate were counted using a dissecting microscope.

(b) Dilutions of virus in Tris-saline Ca 10% were added, in various volumes, to monolayer cultures of BHK21/13 cells in 50 mm. plastic plates. The cultures were maintained at 37° for 1 hour with frequent agitation to allow adsorption to occur. The infecting medium was then removed, and 3 ml. of agar overlay or CMC overlay were added. The plates were then incubated at 37° for 27 hours before the cultures were fixed and stained, as already described and the number of viral plaques estimated.

Assay for Infectious Centres

Monolayer cultures of pseudorabies virus infected BHK21/13 cells were thoroughly washed with Tris-saline and gently trypsinised to give mono-disperse suspensions. 10^2 or 10^3 cells from each of these suspensions were added to 4×10^6 BHK21/13 cells in suspension and dispensed, in 4 ml. ETC, into plastic plates. After incubation at 37° for 3 hours, the growth medium was removed and the cell sheets were overlayed with agar medium. The incubation was continued for a further 27 hours.

The cell monolayers were fixed and stained as before and the number of plaques which had formed were taken as an estimate of the number of infectious centres present in the original inoculum.

One-step Growth Curve of Pseudorabies Virus in a High Serum Tissue Culture System

BHK21/13 cells, suspended in ETC at a concentration of 4×10^6 cells per ml., were infected with pseudorabies virus at a multiplicity of exposure of 5 plaque forming units per cell. After a viral adsorption period of 18 hours at 4°, the cells were then washed with warm Tris-saline Ca 10%, dispensed into plastic plates (4×10^6 cells/plate) in 4 ml. of ETC and incubated at 37°. At hourly intervals from 1-12 hours post infection, at 24 hours and at 48 hours post infection, the cells from 2 plates were suspended in the growth medium and the resultant suspension divided into 2 aliquots. One aliquot was sonicated for five minutes at 60m. amperes in the Dawe Soniclean Generator and the plaque forming units present in the resultant lysate were estimated by the standard assay procedure(a).

This measured the total infectious virus produced.

The second aliquot was centrifuged at 900 g to remove the

cells and the cell-free virus produced was estimated by assaying the plaque forming ability of the supernatant fluid. Infectious centre estimations were also made at hourly intervals up to seven hours post infection.

One-step Growth Curve of Pseudorabies Virus in a Serum Deficient System

BHK21/13 cells, in suspension, were seeded into 50 mm. plastic plates (1.5×10^6 cells per plate) in 10 ml. of ESOH 0.25% Ca medium and incubated at 37° for 4 days. The medium was removed in a sterile fashion and maintained at 37° in an atmosphere of 5% CO_2. The cultures were then inoculated at a multiplicity of exposure of 10 plaque forming units per cell in 0.5 ml. of the medium removed from the cultures. After allowing 1 hour at 37° for viral adsorption, the cell sheet was well washed with warm used medium and 5 ml. of this used medium was added back to the plates. The cultures were incubated once more at 37°. Estimates of the total virus produced per plate were made at hourly intervals from 1-12 hours post infection and at 24 hours post infection by the standard plaque assay technique (a).

Simultaneous Incorporation of Amino Acids during Pseudorabies Virus Infection Examined in(1) A High Serum System and(2) A Serum Deficient System

1. High Serum System.

All the following procedures were at 37°.

Baby bottle cultures of BHK21/13 cells, grown to confluence in EC, were mock infected with 0.5 ml. EC, or infected with pseudorabies virus at a multiplicity of exposure of 20 plaque forming units per cell, in 0.5 ml. EC. After an adsorption period of 1 hour at 37°, the cultures were fed with 10 ml. of EC and the incubation continued. At 2 hours, 4 hours, 6 hours, 8 hours and 10 hours post infection, the medium was removed from 2 uninfected and 2 pseudorabies virus infected cultures, and was replaced by 1 ml. of EC 2.5 mM with respect to ornithine, containing a mixture of $[^{3}H]$ lysine and $[^{14}C]$ arginine. 15 minutes later the radioactively labelled medium was removed and the cultures, washed 2 x with Eagle's medium, were incubated for a further 10 minutes in EC containing 20 x the normal amount of arginine hydrochloride and lysine hydrochloride. The cultures were then chilled and the cell sheet, washed several times with ice-cold PBS A, was suspended in PBS A at 4°.

Cells were disrupted by two cycles of sonication at 60 m. amperes in a Dawe Soniclean generator and the $[^{14}C]$ arginine and $[^{3}H]$ lysine incorporation into 500 μg. of protein, as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), were measured as trichloracetic acid insoluble radioactive material (p.98).

2. Serum Deficient System

BHK21/13 cells, in suspension, were seeded into 50 mm. plates (1.5×10^6 cells/plate) in 10 ml. of ESOH 0.25% Ca medium and incubated at 37° for 4 days. The cultures were then either inoculated with 0.5 ml. of the medium removed from the cultures or with pseudorabies virus at a multiplicity of exposure of 20 plaque forming units per cell (in 0.5 ml. of used medium). After allowing 1 hour at 37° for viral adsorption, 1.5 ml. of the used medium - maintained at 37° in an atmosphere of 5% CO_2 - was added to the plates and the cultures reincubated at 37°. At 2, 4, 6, 8 and 10 hours post infection the cultures were pulsed for 15 minutes in 2.5 ml. EC containing either a mixture of $[^{3}H]$ labelled arginine and $[^{14}C]$ labelled lysine, or a mixture of $[^{3}H]$ labelled valine and $[^{14}C]$ labelled lysine, or a mixture of $[^{14}C]$ labelled valine

and [^{3}H] labelled arginine. In all cases, the medium used was 2.5 mM with respect to ornithine. At each time interval, 2 uninfected and 2 pseudorabies virus infected cultures were labelled with each of these mixtures. After removal of the pulse and washing with warm Eagle's medium, the cultures were chased for 10 minutes with 2 ml. of used medium containing 20 x the normal amount of arginine hydrochloride, lysine hydrochloride and valine. The cultures were then chilled, washed twice with ice-cold PBS A and suspended, using a rubber policeman, in 1 ml. of ice-cold PBS A. After disruption by two cycles of sonication at 60 m. amperes in a Dawe Soniclean Generator, the arginine, lysine or valine incorporation into 200 µg. of protein were measured as trichloracetic acid insoluble radioactivity (p. 98).

Rate of DNA Synthesis During Pseudorabies Virus Infection

(a) High Serum System

Cultures of BHK21/13 cells, grown to confluence in EC in 50 mm. plastic plates containing 3 x 13 mm. coverslips, were infected in 1 ml. EC with pseudorabies virus at a multiplicity of 10 plaque forming units per cell. Cells mock-infected with 1 ml. of EC were used as controls. After an

adsorption period of 1 hour at 37°, a further 1 ml. of EC was added to each plate. At hourly intervals from 2-9 hours post infection one control and one infected culture were pulsed with 10 μC of [^{3}H] thymidine (specific activity 1 C/m-mole) for 1 hour before removing the coverslips from the cultures. Excess radioactivity was removed from these coverslips by 2 rinses in PBS A at room temperature before the cultures were fixed by immersing the coverslips in a mixture of acetic acid and ethanol (3:1) for 10 minutes at 4°. The rate of DNA synthesis per coverslip was measured as the incorporation of [^{3}H] label in 1 hour into trichloroacetic acid insoluble product. Radioactivity was measured by method 4 on p. 98.

(b) Serum Deficient System

The procedures used for radioactive labelling and estimation were identical to those outlined in Section (a) above. However, there were differences in tissue culture and virus infection methods. Those used are described below.

BHK21/13 cells, in suspension, were seeded into 50 mm. plastic plates containing 3 x 13 mm. coverslips (1.5×10^6 cells/plate) in 10 ml. ESOH 0.25% Ca medium and incubated

at 37° for 4 days. The cultures were then either inoculated with 0.5 ml. of the medium removed from the cultures or with pseudorabies virus at a multiplicity of exposure of 10 plaque forming units per cell (in 0.5 ml. of used medium). After allowing 1 hour at 37° for viral adsorption 1.5 ml. of the used medium which had been maintained at 37° in an atmosphere of 5% CO_2 was added to the plates and the cultures reincubated at 37°.

Rate of Protein Synthesis during Pseudorabies Virus Infection

High Serum System

The rate of protein synthesis was estimated as the incorporation of $[^3H]$ label, derived from $[^3H]$ serine, converted into trichloracetic acid insoluble product in 1 hour. The procedure used deviated only from that outlined for the incorporation of $[^3H]$ thymidine in the previous section in the composition of the medium used to label the cells. In this case, 10 μC of $[^3H]$ serine (specific activity 151mC/m-mole) in 2 ml. of EC was used.

In Vivo Labelling Procedures

RNA was labelled in the uridine moiety by incubating newly

confluent roller bottle cultures of BHK21/13 cells at 37° in 20 ml. of EC, 2 μM with respect to uridine and containing various amounts of radioactively labelled uridine. Both [^{3}H] uridine (-5-T) and [^{14}C] uridine (G) were used. The unlabelled uridine was added both to lower the specific activity and to attempt to assist permeation of the labelled material into the cell.

The same basic technique was used to radioactively label the methyl groups present in certain species of RNA. That is to say, newly confluent roller bottle cultures of BHK21/13 cells were incubated at 37° in 20 ml. of EC, 20 mM with respect to ammonium formate and containing various amounts of [^{14}C] methyl methionine. The ammonium formate was added to minimise the incorporation of the radioactive label into the ring structure of the component nucleic acid bases (Davidson, 1965).

In Vivo Chase Procedure

Roller bottle cultures of radioactively labelled BHK21/13 cells were incubated for a further hour in EC medium, 100 μM with respect to non-radioactive uridine, before the cells were harvested.

BIOCHEMICAL METHODS

Pseudorabies virus infected cell cultures used in the following techniques had been infected at a multiplicity of exposure of 20 plaque forming units per cell.

CELL FRACTIONATION PROCEDURE

The following steps were carried out at 4°. Roller bottle cultures of mock-infected or pseudorabies virus infected cells were harvested by scraping into ice-cold PBS A. The cells were spun down at 4° by low speed centrifugation at 900 g and resuspended in PBS A. The centrifugation and resuspension procedure was repeated twice with PBS A and the cells, resuspended in 2 x packed cell volume of ice-cold hypotonic buffer (10 mM-Tris-HCl, pH 7.5, 1.5 mM-$MgCl_2$, 10 mM-KCl and 0.05% bentonite), were allowed to swell for ten minutes at 4°. The cells were then disrupted by gentle homogenisation with a Potter type homogeniser and the nuclei and any undisrupted cells (~1%) removed by centrifugation at 900 g (Penman, Scherrer, Becker & Darnell, 1963). The resultant supernatant fraction will hereafter be termed the 'cytoplasmic fraction and the pellet the nuclear fraction . In certain cases, a further fractionation was undertaken, the cytoplasmic fraction being

spun for 1 hour at 105,000 g in the SW 39 rotor of the Spinco Model L Ultracentrifuge to yield a ribosomal pellet and a non-particulate fraction.

RNA PREPARATIVE PROCEDURES

(a) Two methods were used for extracting the total RNA content of cell fractions.

(i) The cytoplasmic fraction and the non-particulate fractions were deproteinised by agitation for 10 minutes with an equal volume of cold 80% phenol, 0.1% 8-hydroxy-quinoline followed by centrifugal phase separation (Burdon, Martin & Lal, 1967).

(ii) The nuclear fraction and the ribosomal pellet were incubated at 37° for 10 minutes with 2% SDS before deproteinisation by cold phenol extraction. In all cases, the interphases produced during phenol deproteinisation were re-extracted, and the RNA obtained from the pooled aqueous phases, by precipitation at -20° in 2% sodium acetate and 67% ethanol, was collected by centrifugation at 700 g. for 30 minutes at -10°. The RNA samples were extensively washed, dried with ether and dissolved in the presence of bentonite either in SSC or in

50 mM-sodium acetate buffer, pH 5.5.

(b) Low molecular weight 4s RNA for use in hybridisation experiments was prepared as follows. RNA, extracted at 8 hours post infection (as outlined above) from the cytoplasmic fraction of mock-infected or pseudorabies virus infected cells, radioactively labelled between 3 and 8 hours post infection, was further fractionated on Sephadex-G100. The low molecular weight RNA was thus freed from contaminating ribosomal and messenger RNA, and was also resolved into 4s and 5s components. The separated 4s and 5s components were treated with RNase free DNase at 10 µg./ml. in 2 mM-$MgCl_2$ for thirty minutes at 37° and the enzyme added removed by deproteinisation with 90% phenol. The aqueous phase, recovered by centrifugation at 1000 g and dialysed against 2 changes of SSC for 2 hours and 18 hours respectively, was stored frozen at -70°.

(c) Preparation of sRNA

The following steps were carried out at 4°. Roller bottle cultures of mock-infected and pseudorabies infected cell cultures were harvested in versene at 6.5 hours after infection, spun down by low speed centrifugation at 900 g and washed 3 x with Tris-saline, pH 7.5, solution and

once with 20 mM-Tris-HCl buffer pH 7.5. Prior to use, the preparations were stored at -70° suspended in a volume of 20 mM-Tris-HCl pH 7.5, 5mM-$MgCl_2$, 0.05% bentonite equal to 3 x the packed cell volume. Complete cell disruption was accomplished by repeated cycles of freezing and thawing or by sonication at 1.5 amperes in an M.S.E. sonicator. The cell lysate so produced was made 50 mM with respect to Tris-HCl buffer pH 7.5, 0.3M with respect to sucrose, 25 mM with respect to KCl and 4 mM with respect to $MgCl_2$ and centrifuged at 105,000g for 2 hours in the 40 head of the Spinco Model L Ultracentrifuge. RNA was extracted as follows by a modification of the method of Hoageland, Stephenson, Scott, Hecht & Zamecnik (1958). The pH of the resultant non-particulate fraction was carefully adjusted to 5.0 by the addition, with controlled stirring, of 1 M acetic acid and the flocculent precipitate which appeared was collected by centrifugation for 15 minutes at 700 g. After dissolving it in 20 mM-Tris-HCl pH 7.5, 4 mM-$MgCl_2$, 0.05% bentonite, an equal volume of 90% phenol was added and the emulsion shaken for 10 minutes before centrifugation at 1,000 g to give phase separation. The aqueous layer was carefully

removed and the protein interphase and phenol layer re-extracted as before with 20 mM-Tris-HCl, 4 mM-$MgCl_2$. The combined aqueous layers, made 2% with respect to sodium acetate and 67% with respect to ethanol, were placed at -20° to facilitate precipitation of the RNA. The precipitate of aminoacyl-tRNA was collected by centrifugation at 700 g for 30 minutes at -10°. Amino acid moieties were then dissociated from the tRNAs by dissolving the preparation in 1.8 M-Tris-HCl buffer pH 8.5 and incubating the solution at 37° for thirty minutes (Sarin & Zamecnik, 1964). After precipitation as before, the RNA was renatured by the process outlined by Lindahl, Adams & Fresco (1966). This involved dissolving the RNA in a minimum volume of 20 mM-Tris-HCl buffer pH 7.5, 15 mM-$MgCl_2$, 0.01% SDS and maintaining the solution for 5 minutes at 60° before allowing it to cool slowly to 4°. The solution was then dialysed, at 4°, against 2 litres of 20 mM-Tris-HCl pH 7.5, 1 mM-$MgCl_2$ for 2 hours and 24 hours respectively. This dialysate residue is hereafter termed sRNA preparation. The average sRNA yield from 10 roller bottle cultures, calculated from measurements of the absorbance of the product at 260mμ, was 2 mg.

(d) Preparation of Aminoacyl-tRNA

The preparation of aminoacyl-tRNA from sRNA preparations was effected enzymically as described on p.101

DNA PREPARATIVE PROCEDURES

(a) Preparation of Cellular DNA

By a modification of the method of Marmur (1961).

Roller bottle cultures containing approximately 4×10^8 BHK21/13 cells per bottle were harvested in versene solution, spun down at 4° by low speed centrifugation (700 g.) and washed several times with SSC by repeated resuspension and centrifugation. The cells were finally suspended in 2 x packed cell volume of SSC and the suspension made 2% with respect to SDS. The resultant viscous solution was rolled gently at 20° for 1 hour before sodium perchlorate was added to a concentration of 1 M. Deproteinisation was effected by shaking the solution for 30 minutes at 4° with an equal volume of a chloroform-isoamyl alcohol mixture and centrifuging the resultant emulsion to give phase separation. After removing the aqueous phase, the protein interphase was re-extracted with an equal volume of SSC. The DNA, spooled from the combined aqueous phases by the addition of 2 volumes of

ethanol, was washed with alcohol, allowed to dry and dissolved in the minimum volume of SSC/100. RNA contamination was removed by incubating the DNA, now in SSC, with 20 μg. /ml. of boiled pancreatic ribonuclease for 60 minutes at 31°. After further deproteinisation with phenol, the DNA was isolated as already described and incubated for 2 hours at 37° with 50 μg. /ml. of self digested pronase as recommended by Gillespie & Spiegelman (1965). The pronase was removed by deproteinisation with phenol and chloroform - isoamyl alcohol mixture in turn, and the DNA recovered by ethanol precipitation. This DNA preparation which exhibited no ribonuclease activity, as monitored using radioactive RNA as substrate, was preserved at 4° in SSC containing a drop of chloroform.

(b) Preparation of Pseudorabies Virus DNA

Combined preparations of pseudorabies virus containing a total of 5×10^{12} virus particles were suspended in PBS A, 1 mM with respect to $MgCl_2$ and incubated with RNase and DNase at 50 μg. per ml. for 60 minutes at 37°. The virus particles, pelleted by centrifugation at 38,000 g for 60 minutes, were resuspended in a minimal volume of SSC and, after the

addition of SDS to a final concentration of 2% the preparation was incubated at 42° for 30 minutes. The concentration of the resultant viscous solution was altered to 1 M with respect to sodium perchlorate and slowly agitated at 4° for about 20 minutes. An equal volume of a chloroform-isoamyl alcohol mixture (in the proportion of 50 volumes of chloroform to one volume of isoamyl alcohol) was added and the solution gently agitated for a further 10 minutes. The aqueous phase was then recovered by centrifugation at 1000 g, the chloroform layer and interphase being reextracted with SSC. After combining the aqueous phases, the viral DNA was gently spooled out (by the addition of 2 volumes of ethanol). It was then washed with ethanol, allowed to dry and was stored dissolved in SSC/50 overnight before being banded in the SW 65 head of the Spinco Model L Ultracentrifuge, in 3 aliquots, by equilibrium density gradient centrifugation using the following technique.

120 μg. of DNA was mixed with 2.65 g. CsCl dissolved in 2 ml. of water and spun at 10° in the SW 65 head of the Spinco Model L Ultracentrifuge at 30,000 rev./min. for 54 hours. On completion of the run, the bottom of the tube

was punctured with a pin and 2 drop fractions were collected across the gradient. The fractions were diluted with 0.5 ml. of SSC to permit location of the DNA band by its absorbance at 260 mμ.

The bands of viral DNA so obtained were pooled and the DNA respooled by the addition of 2 x the volume of ethanol. After drying the DNA was dissolved in 0.15 ml. of SSC/100. The solution was then made up to a concentration of SSC for storage, the yield at this stage being 280 μg.

Preparation of Denatured DNAs

The pseudorabies virus DNA obtained as above was diluted to 50 μg. per ml. with water as a preliminary to denaturation. This was effected by maintaining the DNA in SSC/50 at 100° for 10 minutes and quick cooling in ice. The completeness of the process was monitored by hyperchromic changes.

Cellular DNA was denatured by alkali in SSC/50 at a concentration of 100 μg./ml. The pH of the solution was adjusted to 13 and after standing for some 10 minutes, the solution was neutralized. Denaturation was monitored by following the increase in absorbance at 260 mμ.

ESTIMATION PROCEDURES

(a) Determination of Radioactivity

Radioactive material was assayed in a Nuclear Chicago liquid scintillation spectrometer. The efficiency of counting a single isotope was determined using the channels ratio counting mode to estimate the degree of quenching. Application of quench correction factors also permitted simultaneous estimation of two isotopes of different energy emission spectra. In this case, the degree of quenching was measured either by the external counting mode or by the channels ratio counting mode using the isotope with the higher energy emission spectrum, and assuming necessarily that the correction factors so obtained were a true estimate of the quenching of both isotopes. Such an assumption would not be justified if coloured compounds were present in the system. To maximise efficiency of counting, materials such as water, sodium chloride and ammonium formate which lead to quenching, were removed by one of the following methods and the samples were either counted immersed as thin layers on translucent filters or coverslips or dissolved in the scintillation fluid.

1. Acid insoluble material precipitated from the samples by the addition of 50% trichloracetic acid to a final concentration of 5% was collected by suction filtration onto Millipore filters. The filters so loaded were washed with 5% trichloracetic acid, dried for 2 hours at 60° and immersed in a vial in 10 ml. of toluene based scinstant. In certain cases, 50 μg. of bovine serum albumin were added as coprecipitant.

2. Samples in 0.1 ml. aliquots were spotted on Chromedia glass filter discs and dried at 60° for 2 hours before immersion in 10 ml. of toluene based scinstant.

3. Samples, already dispensed into scintillation vials, were heated at 160° for 3 hours to evaporate or sublime any quenching agents. The residue was dissolved, by heating at 56° for five minutes, in 10.5 ml. of a (20 v/v) mixture of toluene based scinstant and hyamine hydroxide.

4. All processes at 4°. Acid insoluble material in BHK21/13 cells grown on glass coverslips and fixed with a mixture of acetic acid and ethanol was precipitated by exposure of the coverslips to 5% trichloracetic acid for 10 minutes. The coverslips were then given three 10 minute washes with

distilled water and one with ethanol and, after drying, immersed in vials containing 10 ml. of toluene scinstant.

(b) Determination of Salt Concentration

This method was used both for the determination of the concentration of ammonium formate and for the determination of sodium chloride concentration. Concentrations were determined by measurement of the refractive indices in an Abbe refractometer which had been calibrated using solutions of known salt concentration.

(c) Determination of Extinction

Absorbance at 260 mμ was measured using a Unicam SP 500 spectrophotometer with matched silica microcells.

PREPARATIONS OF CRUDE ENZYME EXTRACTS

1. Used as a source of methylases

The following steps were carried out at 4°. Roller bottle cultures of mock-infected and pseudorabies virus infected BHK21/13 cells were harvested at 5 hours post infection by scraping into ice-cold PBS A using a rubber policeman. The cells were then washed by centrifugation and resuspension twice with PBS A and once with 10 mM-2'-mercapto ethanol,

20 mM-Tris-HCl buffer pH 8.0 (Burdon, Martin & Lal, 1967). The pellets were suspended in 4 x the packed cell volume of this buffer and 10 minutes later the cells were disrupted by 25 strokes with a Potter type homogenisor. The resultant lysate formed the crude enzyme extract, the protein concentration of which was estimated by the method of Lowry et al., (1951).

2. Used as a source of aminoacyl transferase

The following steps were carried out at 4°. Roller bottle cultures of mock-infected and pseudorabies virus infected BHK21/13 cells were harvested at 6.5 hours post infection by scraping into ice-cold PBS A, spun down by low speed centrifugation at 900 g and resuspended in PBS A. This washing procedure was repeated with PBS A and with TEKM buffer (20 mM-Tris-HCl pH 7.5, 15 mM-KCl, 10 mM-2'-mercapto ethanol, 5 mM-$MgCl_2$). The cells, suspended in 4 x packed cell volume of this buffer, could be stored for at least 1 month at -70° without loss of enzymic activity. Normally, the cells were immediately disrupted by homogenising the suspension for 2 minutes in a Nelco blade

homogeniser and the resultant lysate was spun at 105,000 g for 2 hours in the SW 39 head of the Spinco Model L Ultracentrifuge. Dialysis of the non-particulate fraction for two one hour intervals against three litres of 20 mM-Tris-HCl buffer pH 7.5, 15 mM-KCl, 5 mM-$MgCl_2$ removed heavy metal ions and endogenous free amino acid. The dialysis residue constituted the crude aminoacyl transferase extract, the protein concentration of which was estimated, before use, by the method of Lowry et al., (1951).

IN VITRO ENZYMIC REACTIONS

(a) Aminoacyl-tRNA Transferase Reactions

These enzymic reactions were utilized in the preparation of radioactively labelled aminoacyl-tRNAs essentially by the method of Muto, Miura, Hayatsu & Ukita (1965). A 1.5 ml. reaction mixture contained the following components: 100 μmoles Tris-HCl buffer pH 7.6; 25 μmoles $MgCl_2$; 10 μmoles ATP; 2 μmoles CTP; 50 μmoles KCl; 1 mg. isolated sRNA; 5 μc[^{14}C]or 50 μc[^{3}H]labelled amino acid plus unlabelled amino acid to a final concentration of 200 mμmoles/ml., the 19 remaining amino acids at a concentration of

140 mμmoles/ml. and enough crude aminoacyl-tRNA transferase (approx. 2 mg. of protein) to obtain maximum formation of aminoacyl-tRNA. The reaction was initiated by the addition of the enzyme preparation and the mixture incubated for 17 minutes at 37°. After chilling in ice, 0.5 ml. of water, 0.25 ml. of IM-KCl and 4 ml. of freshly distilled 90% phenol, 0.1% with respect to 8-hydroxy-quinoline, were added and the resultant emulsion shaken for 2 periods of 5 minutes duration, 2 ml. of 0.5 M-sodium acetate buffer pH 5.1 being added in the interim. The aqueous phase was recovered by centrifugation at 16,000 g , made 2% with respect to sodium acetate and 67% with respect to ethanol, and placed at -20°. Aminoacyl-tRNA which precipitated under these conditions was collected by centrifugation at 700 g for 30 minutes at -10°, washed by resuspension and centrifugation in turn in 0.5 M-sodium acetate buffer pH 5.1, ethanol/0.5 M-sodium acetate pH 5.1 (2 v/1v), ethanol, ethanol/ether (equal volumes) and ether (Ishida & Miura, 1965). After removing the ether in a stream of nitrogen, the aminoacyl-tRNA was dissolved in 1 ml. of 0.1 M-sodium acetate buffer pH 5.5, 2 mM with respect to EDTA and stored

at -70°. The degree of amino acid acceptance was estimated as acid precipitable radioactive material.

(b) Methylase Reactions

Conditions used in an attempt to methylate [^{3}H] uridine labelled low molecular weight RNA from pseudorabies virus infected cells were essentially those described by Hurwitz, Gold & Anders (1964).

A 1 ml. reaction mixture contained the following components:- 5 μmoles 2'-mercapto ethanol, 3 μmoles ATP, 20 μmoles Tris-HCl buffer pH 8.0, 20 μmoles $MgCl_2$, 10 μg. heparin, 20 mμmoles methionine, 200 μg. [^{3}H] uridine labelled low molecular weight RNA and crude cell extract (containing a total of 2 mg. protein) as a source of methylating enzyme. The reaction was initiated by the addition of the enzyme preparation and the mixture incubated for 40 minutes at 36°. After chilling in ice, an equal volume of freshly distilled 90% phenol, 0.1% with respect to 8-hydroxy quinoline, was added and the resultant emulsion shaken for 2 periods of 5 minutes duration, 2 ml. of 20 mM-Tris-HCl pH 8.0, 10 mM-2'-mercapto ethanol being added in the interim. The aqueous phase was recovered by centrifugation, made

2% with respect to sodium acetate, 67% with respect to ethanol and placed at -20°. The RNA which precipitated was collected by centrifugation at 700 g for 30 minutes at -10°, washed by centrifugation and resuspension with ethanol and ether in turn and dissolved in SSC. Excess ether was removed in a stream of nitrogen and the RNA was stored frozen at -70°.

T_1 RNase Digestion

500-1000 μg. of mixed control and infected differentially labelled aminoacyl-tRNA dissolved in 1 ml. of 0.1 M-sodium acetate pH 5.5, 2.0 mM with respect to EDTA (Sato & Egami, 1957), was incubated at 37° for 60 minutes with 500 units of T_1 RNase (Ishida & Miura, 1965). The mixture was then removed into ice and added to 5 ml. of ice-cold 0.1 M-sodium acetate buffer pH 5.5, 6 ml. of freshly distilled 90% phenol, 0.1% with respect to 8-hydroxy-quinoline, was added and the resultant emulsion shaken for 10 minutes and spun at 1000 g. for 10 minutes to give phase separation. After re-extraction of the interphase, the pooled aqueous phases, containing aminoacyl-oligonucleotides, were diluted to 15 ml. with ice-cold distilled water as a preliminary to DEAE cellulose

chromatography.

CHROMATOGRAPHIC PROCEDURES

(a) Gel Filtration on Sephadex-G100

RNA preparations, dissolved in SSC, were applied in a maximum volume of 0.5 ml. to a 1.5 x 80 cm. column of Sephadex-G100 (Galibert et al., 1965) which had been prepared by the method of Flodin (1962) and previously equilibrated with SSC. The RNA was eluted from the column with SSC at 16° under a slight gravitational head. In certain cases, other buffers were used, namely, 50 mM-sodium acetate pH 5.5; 10 mM-Tris-HCl pH 7.4, 20 mM with respect to $MgCl_2$, 1 mM with respect to EDTA; and 10 mM-Tris-HCl pH 7.4, 1 mM with respect to EDTA. In all cases, the eluate was collected in 1 ml. fractions which were immediately placed at 4° and subsequently measured both for absorbance at 260 mμ and for radioactivity. The radioactive material in the fractions was estimated either as trichloracetic acid insoluble radioactivity or directly on glass filters.

(b) DEAE Cellulose Column Chromatography

DEAE cellulose powder was suspended in distilled water and the fines removed by several decantations.

The remainder was washed with acid and alkali as described by Peterson & Sober (1956), thoroughly rinsed with water and adjusted to pH 5.5. It was then resuspended in 10 mM-ammonium formate pH 5.5 and stored at 4°.

Chromatography of aminoacyl-oligonucleotides was performed at 4° to prevent cleavage of the bond between the amino acid and the polynucleotide. The aminoacyl-oligonucleotide fragments produced by T_1 RNase digestion of 500 μg., say, of mixedly labelled tRNA were absorbed in 15 ml. to a 1 x 5 cm. column of DEAE cellulose which had been packed at 20° under a pressure of 5 lb./sq. in. and equilibrated at 4° with 10 mM-ammonium formate pH 5.5 (Ishida & Miura, 1965). Free amino acid in the preparation was removed by washing the column with 80 ml. of 10 mM-ammonium formate pH 5.5 and the oligonucleotides were fractionated using an increasing linear concentration gradient of ammonium formate buffer pH 5.5 (10 mM to 0.6 M) (Herbert, Smith & Wilson, 1964). The flow rate averaged 1.5 ml. per minute and 3 ml. fractions of the eluate were collected. Estimations of the salt concentration, the absorbance at 260 mμ and the total radioactivity of $[^{14}C]$

and [^{3}H] were made on these fractions by the standard evaporation method (p. 98).

(c) MAK Column Chromatography

MAK columns (Mandell & Hershey, 1960; Sueoka & Cheng, 1962) were prepared by sequentially packing 20 ml. of MAK and 4 ml. of Kieselguhr under pressure of 5 lb. per sq. in. to give a column consisting of one layer of 2 x 5 cm. MAK and one 2 x 1.5 cm. of Kieselguhr, the latter being the upper layer. Fine glass beads were then layered on to the surface of the Kieselguhr and the column washed with 200 ml. of 0.10 M-NaCl, 50 M-sodium phosphate buffer pH 6.25.

500 μg. of mixed control and infected differentially labelled aminoacyl-tRNA was applied at a concentration of 20 μg. per ml. in 0.15 M-NaCl, 50 mM-sodium phosphate buffer pH 6.25 at a rate of 1 ml. per minute to a MAK column prepared as above. Elution was effected at pH 6.25 and 16° by a linear gradient of increasing NaCl concentration. The flow rate, maintained by a peristaltic pump, was 1 ml. per minute. 3 ml. fractions were collected and the NaCl concentration, absorbance at 260 mμ. and the radioactive material precipitable with 5% trichloracetic acid (both [^{3}H]

and $\left[^{14}C\right]$) were estimated by methods previously outlined.

(d) Reverse Phase (Type II) Column Chromatography

Reverse phase (type II) column chromatography was carried out essentially by the method of Weiss & Kelmers (1967). The organic phase used was a 5% v/v mixture of methyl,tricaprylyl,ammonium chloride (Aliquat 336) in tetrachloro, tetrafluoropropane (Freon 214). The chromatographic column packing consisted of 30 ml. of the organic phase thoroughly mixed at room temperature with 50 g. of hydrophobic diatomaceous earth (Chromosorb W). The packing was left at room temperature for seven days to allow equilibration of the phases and thereafter stored as a slurry in an aqueous solution (0.25 M-NaCl, 10 mM-sodium acetate buffer pH 4.5, 10 mM-$MgCl_2$) which had been saturated with Freon 214. All aqueous solutions in the chromatographic experiments were saturated with Freon 214 to prevent dilution of the Freon 214 in the column. The chromatographic column was prepared at 37° by filling a 0.6 x 180 cm. column with an aqueous solution of the above buffer. The slurry was slowly poured into the column, allowed to settle and compacted by the application of a pressure of 5 lb./sq. in.

Such a column permitted a free flow of the mobile aqueous phase and could be used repeatedly for many experiments by regeneration with 1 M-NaCl after each run. The chromatographic experiments were performed at 16°, the column having been washed with 100 ml. of the starting buffer before the aminoacyl-tRNA (1 mg. - 1.5 mg.) was applied to it in a volume of 1 ml. At this NaCl concentration the aminoacyl-tRNA was tightly bound to the chromatographic matrix. Generally, mixed control and infected, differentially labelled aminoacyl-tRNA preparations were applied. A linear gradient of 0.25-0.75 M-NaCl, containing 10 mM-sodium acetate buffer pH 4.5 and 10 mM-$MgCl_2$ was used to elute the tRNAs from the column at a flow rate of 1 ml. per minute. The eluate was collected in 2 ml. fractions which were measured for absorbance at 260 mμ and trichloracetic acid precipitable radioactivity (both $[^3H]$ and $[^{14}C]$) by the standard method (p. 98).

DNA - sRNA HYBRIDISATION

Hybridisation was carried out by a modification of the method of Gillespie & Spiegelman (1965) which consisted of immobilising

DNA by binding it to nitrocellulose membrane filters, hybridising RNA to the fixed DNA and removing unpaired RNA and RNA complexed over short regions.

1. Immobilization

Schleicher and Schuell membrane filters were prepared by presoaking the filters for 1 minute with 6 x SSC and washing each side with 10 ml. of 6 x SSC. Denatured DNA solutions at a concentration of 10 μg. per ml. in 6 x SSC were slowly passed through the filters which were then thoroughly washed with 100 ml. of 6 x SSC by suction filtration. The DNA filters were dried for 4 hours at room temperature and at 65° for 18 hours.

2. Hybridisation

Hybrids were formed by immersing the DNA filters in scintillation vials containing 5 ml. of $[^3H]$ uridine labelled 4s RNA, in 6 x SSC. Annealing was carried out, without shaking, at 65° for 18 hours after which time the vials were cooled quickly.

3. Elimination of "Noise"

The filters were removed from the hybridisation mixture and each side was washed with 50 ml. of 2 x SSC by suction

filtration. Uncomplemented RNA was destroyed by immersing the filters for 1 hour at 20° in 5 ml. of 2 x SSC containing 20 μg. per ml. of boiled pancreatic RNase. The filters were then rewashed on each side as before, dried and counted immersed in toluene scinstant.

RESULTS

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RESULTS

The experimental observations to be reported are, for convenience, divided into 5 general sections.

1. The description of optimal conditions for growth, storage and assay of pseudorabies virus.
2. General characteristics of pseudorabies virus infection in BHK21/13 cells.
3. The metabolism of pulse-labelled low molecular weight RNAs in non-infected and pseudorabies virus infected BHK21/13 cells.
4. The genetic origin of 4sRNA in pseudorabies virus infected BHK21/13 cells.
5. The composition of aminoacyl-tRNA populations in non-infected and pseudorabies virus infected BHK21/13 cells.

1. DESCRIPTION OF OPTIMAL CONDITIONS FOR GROWTH, STORAGE AND ASSAY OF PSEUDORABIES VIRUS

(a) Effect of Multiplicity and Growth Medium on Virus Yield.

When BHK21/13 cells were exposed to pseudorabies virus at varying multiplicities for 1 hour

at 37° and the virus then permitted to multiply, in either EC or ETC growth medium, until typical cytopathic effect was visible in all cells, the yield of virus obtained varied substantially. This variation could be correlated to the multiplicity of infection and to the growth medium used. Table (3) shows the results of 8 experiments in which different multiplicities of the same pseudorabies virus preparation were used for infection. The results indicate an almost 20 fold increase in yield from the cultures infected at a multiplicity of exposure of 0.0025 plaque forming units per cell, compared to that obtained from those infected at a multiplicity of 10 plaque forming units per cell. Marginally higher viral yields (2 fold) were also obtained by incubating the infected cells in ETC rather than EC growth medium. It would therefore seem, that uninfected cells produced a substance required for viral growth and that this substance was supplied by the provision of Tryptose phosphate broth. Preformed purine and pyrimidine nucleosides are the obvious candidates. On the basis of these results, multiplicities of infection of less than 1 plaque forming unit per 300

Table 3.

Growth Medium	Multiplicity of Infection PFU/cell	Viral Yield $\log_{10}$ PFU/culture	Incubation Period (hrs.)
ETC	0.0025	8.35	36
ETC	0.025	8.01	27
ETC	1.00	7.14	27
ETC	10.00	6.95	27
EC	0.0025	8.07	36
EC	0.025	7.47	27
EC	1.00	7.10	27
EC	10.00	6.80	27

Effect of Growth Medium and Multiplicity of Infection on the Yield of Pseudorabies Virus from BHK21/13 Cells.

Confluent monolayer cultures of BHK21/13 cells in 50 mm. plastic plates were infected with pseudorabies virus in 0.5 ml. of EC or ETC at the multiplicities of exposure indicated. After adsorption for 1 hr. at 37° the cultures were fed with a further 3.5 ml. of the corresponding medium and incubated at 37° for the times shown. The cells were harvested and the virus extracted by sonication. The virus yield, expressed at $\log_{10}$ PFU per culture, was estimated by the standard plaque assay technique.

cells were used for the routine growth of high titre stocks of pseudorabies virus and ETC was selected as the growth medium. This technique resulted in a particle infectivity ratio of less than 5:1.

(b) Effect of Temperature on Virus Yield.

The effect of temperature on the multiplication of pseudorabies virus was estimated by measuring the yield of virus obtained from cultures incubated at 31° or 37° throughout the viral adsorption and multiplication phases. In all cases, the cultures were infected at a multiplicity of exposure of 0.003 plaque forming units per cell, provided with ETC growth medium and harvested when every cell showed evidence of the typical viral cytopathic effect (27 hours post infection at 37°, 48 hours post infection at 31°). At 37°, each cell yielded an average of 200 plaque forming units while at 31° the average viral yield per cell was 250 plaque forming units. Despite this marginal increase in the yield at 31°, 37° was routinely used for the preparation of pseudorabies virus stocks since an incubation period of 27 hours was more convenient and

more economical in terms of incubator space.

(c) Effect of Storage Conditions on Viral Infectivity.

The effect of storage, in Tris-saline Ca. 10%, on the viability of pseudorabies virus is shown in Table (4). Storage for 8 days at 4° or -70° had no effect on the titre of infectious virus in the preparations. After 2 months at -70° the titre had dropped by a factor of 2, but this is probably not significant. The virus is, therefore, relatively stable and can be safely stored for long periods at -70° and for at least 1 week at 4°.

(d) Effect of Assay Conditions on Accuracy of Infectivity Determinations.

The standard plaque assay used in the following experiments, unless otherwise stated, was that previously described (p. 78).

The effect of altering the infecting conditions in the plaque assay is shown in Table (5). The pseudorabies virus suspensions assayed under each of the conditions listed were adjusted so that the same total virus inoculum was present in all the infected cultures. The results indicate that, when infecting monolayer

Table 4.

Conditions of Storage	Titre of Infectious Virus $\log_{10}$ PFU/ml.
0 days	8.22
8 days at 4°	8.18
8 days at -70°	8.30
60 days at -70°	8.00

Effect of Storage on Pseudorabies Virus.

Identical suspensions of pseudorabies virus in Tris-saline Ca 10% were stored for various periods of time at either -70° or 4°, before the titre of infectious virus was estimated by the plaque assay technique.

Table 5.

Infecting Procedure	Estimate of Titre ($\log_{10}$ PFU/ml.)	
	I	II
Infection in Suspension	8.7	10.25
Infection in Monolayers (1 ml.)	8.19	9.0
(0.5 ml.)	8.24	9.10
(0.2 ml.)		9.56

Effect of Mode of Infection on the Efficiency of the Plaque Assay Procedure

Two separate preparations of pseudorabies virus were used, dilutions being made in Tris-saline Ca 10%. The inoculum was either incubated on monolayer cultures in 50 mm. plastic plates in the volume indicated for 60 mins. at 37° or cells infected in suspension were agitated under similar conditions. The inoculum was removed and the monolayer cultures washed and overlayed with a standard agar overlay medium or the suspended cells were allowed to adhere to plastic plates for 3 hrs. before similar treatment. 27 hrs. later the number of plaques formed per plate were counted and the figures shown give the titres established from the average of several plates.

cultures, it was obviously advantageous to use an 0.2 ml. infecting volume in preference to one of 0.5 ml. or 1 ml. However, when infecting coverslip cultures in plastic plates, an infecting volume of 1 ml. was routinely used since the repeated tipping of the plate essential for even infection when using an infecting volume of 0.2 ml., is not desirable. Care was taken to ensure that any such discrepancy was accounted for in the calculation of the multiplicity of infection. Infection in suspension appears to be the most efficient assay procedure and this was therefore routinely used.

Several different overlay procedures were also investigated, namely CMC medium, agar overlay medium, ETHu and ETC containing heparin at 25 μg./ml. It was observed with both CMC medium and ETHu that, at 24 hours post infection, the plaques were not discrete but diffuse and streaked but, with both the agar overlay medium and the ETC containing heparin, discrete plaques were produced. By 48 hours, the plaques produced on the plates with ETC containing heparin also showed signs of streaking. At this time, discrete plaques were still

present on those cultures which had been overlayed with agar medium. The agar overlay method was, therefore, used although this necessitated allowing 2 - 3 hours for the cells to stick to the plates before applying the overlay.

To determine the ideal incubation period for plaque development, identical pseudorabies virus suspensions were assayed by the standard plaque assay technique and the plaques allowed to develop at 37° for 27 or 48 hours. The plaques at 48 hours were larger than those at 27 hours but the average number of plaques produced per plate were identical, within the limits of experimental error. It was, therefore, possible to count a greater number of plaques per plate at 27 hours and for this reason this time interval was chosen for routine use.

The development of the plaque assay system just described and the availability of high titre stocks of pseudorabies virus facilitated the investigation of infection of BHK21/13 cells with the virus under conditions in which every cell was infected. The

process of infection (in BHK21/13 cells) with the newly isolated virus strain was characterised, so that meaningful comparisons could be drawn with previously reported data and so that the optimal experimental conditions were used in biochemical investigations comparing low molecular weight RNAs in non-infected and pseudorabies virus infected BHK21/13 cells.

2. GENERAL CHARACTERISTICS OF PSEUDORABIES VIRUS INFECTION OF BHK21/13 CELLS

(a) Single Step Growth Curve of Pseudorabies Virus in a High Serum System.

Cell suspensions were inoculated at 4° with 5 plaque forming units of pseudorabies virus per cell and, after 18 hours at 4,° dispensed into plastic dishes. The infected cultures were grown in ETC at 37° and samples removed at selected times after infection. Increases in total infectious virus (growth medium + cells) and in the infectious virus already released into the growth medium (expressed as log units) are plotted against time after infection (Fig. 3). The variation, with time, of the number of infectious centres present per 10^6 cells is also shown in this figure. The results, with reference to total virus production, show that in 1 hour at 37° the

Figure 3.

One-step Growth Curve of Pseudorabies Virus in BHK21/13 Cells in a High Serum Tissue Culture System.

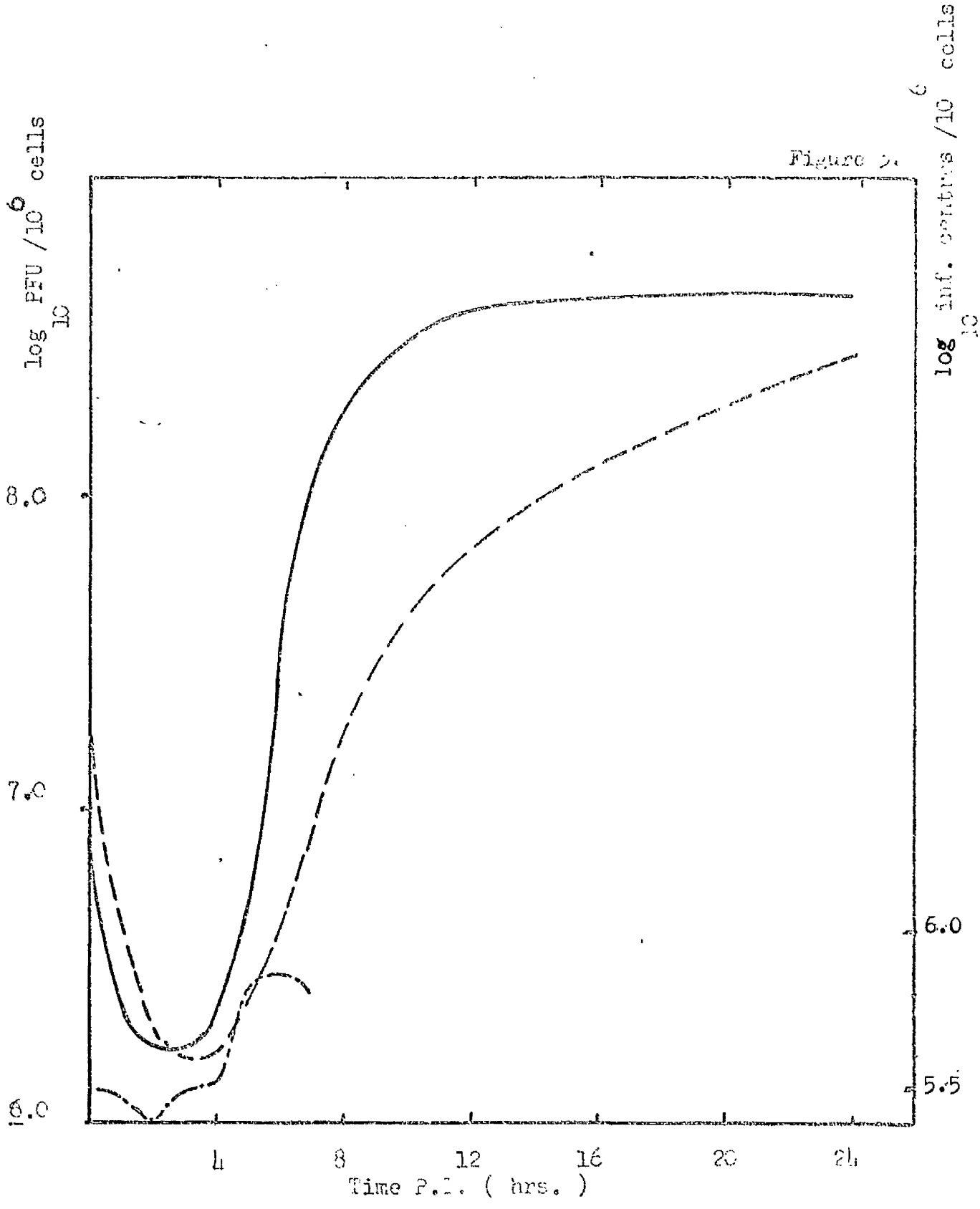
4×10^6 cells in suspension, in ETC, were infected at a multiplicity of exposure of 5 PFU per cell with pseudorabies virus and the suspension maintained at 4° for 18 hrs. The cells were then grown in 4 ml. of ETC at 37° in plastic dishes containing 4×10^6 cells per dish. At selected times P.I., 2 cultures were separately estimated for total infectious virus produced, for cell free virus produced and for infectious centres. Standard assay procedures were used. The values obtained from the duplicate cultures were averaged and the infectivity titres, expressed as logarithms of PFU per 10^6 cells originally plated, plotted against time P.I. Infectious centres were expressed as logarithms of the number of infected cells per 10^6 cells harvested.

———— Total infectious virus.

– – – Cell-free virus.

–.–.– Infectious centres.

Figure 2.


log PFU /10^6 cells
log Inf. centres /10^6 cells
8.0
7.5
6.0
5.5
4
8
12
16
20
24
Time P.I. (hrs.)

virus, which had been electrostatically adsorbed at 4°, had penetrated the cells and that infectious (progeny) virus was first produced between 4 and 5 hours post infection. Exponential growth then took place until 10 hours post infection, after which time no significant increase in the infectious virus titre was observed. During the exponential phase of 4 hours' duration, a one thousandfold increase in the number of plaque forming units took place. The average yield of total virus per cell was 400 plaque forming units, while the average yield per infectious centre was 1,200 plaque forming units. Similar results have been reported by Kaplan & Vatter (1959) using an RK cell system.

Examination of the plot of infectious virus present in the growth medium against time indicates that from 5-10 hours post infection virus was released from the cells into the surrounding medium. The rate of release was relatively constant over this period but was significantly slower than that of the virus production within the cells. This process continued at a slower

rate up till 24 hours post infection, at which time the greater proportion of the infectious virus was no longer cell associated but found in the growth medium. Typical viral cytopathic effect - a generalised rounding up of cells - was first observed at about 5 hours post infection.

(b) Single Step Growth Curve of Pseudorabies Virus in Low Serum System.

The production of infectious virus in cells previously deprived of serum was also examined. "Stationary" cultures were inoculated with 10 plaque forming units of pseudorabies virus per cell in 'used' medium and unadsorbed virus was removed by washing, after an incubation period of 1 hour at 37°. The infected cultures were grown in 'used' ESOH $\frac{1}{4}$% Ca. and samples removed at hourly intervals up to 12 hours post infection, at 24 hours and 30 hours post infection. Increases in total infectious virus (expressed in log units) are plotted against time after infection (Fig. 4).

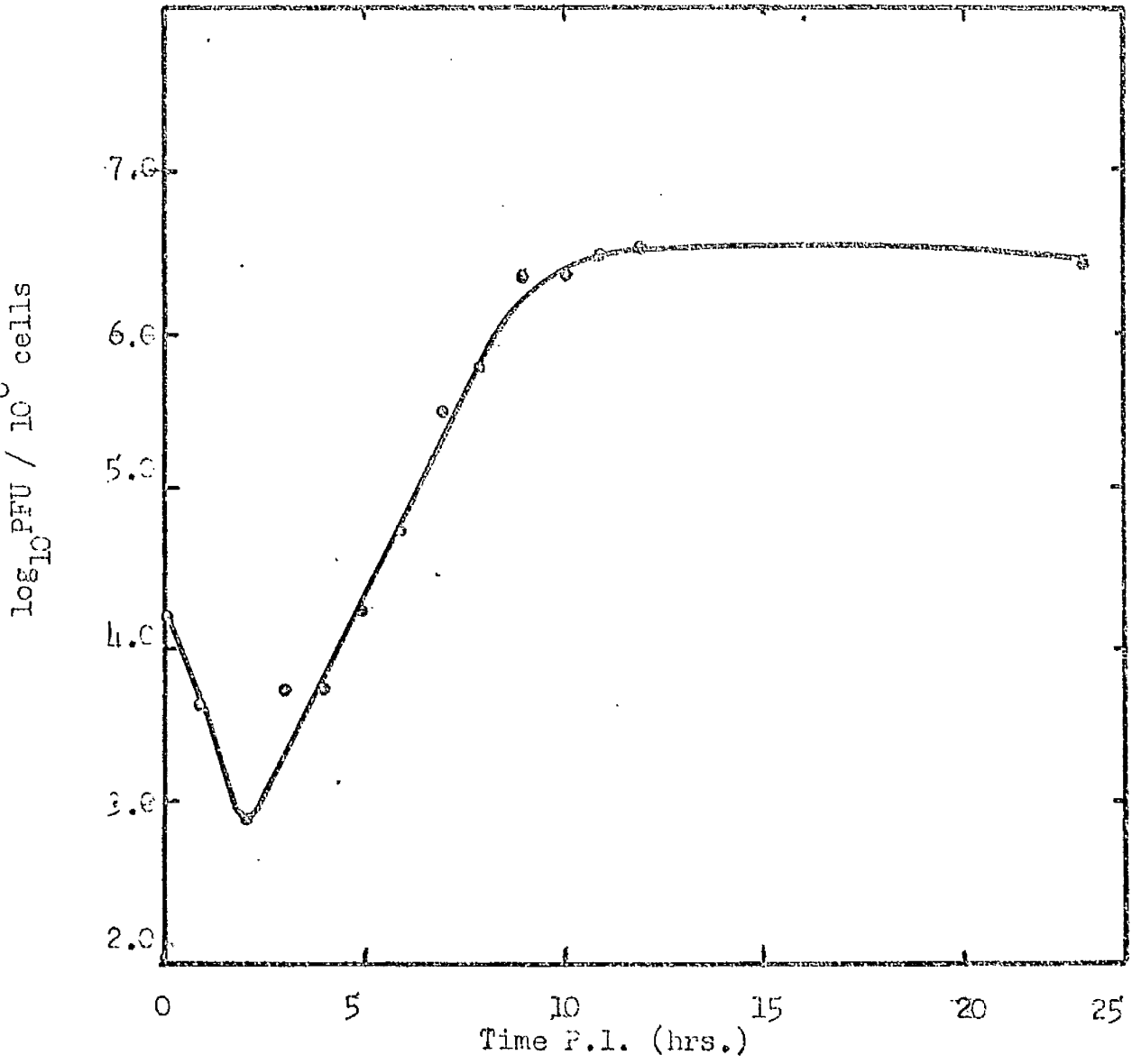
The results indicate that the viral inoculum had been taken up by 1 hour post infection. From 5-9 hours infectious virus production was exponential, a 200 fold

Figure 4.

One-step Growth Curve of Pseudorabies Virus in BHK21/13 Cells in a Serum Deficient Tissue Culture System.

1.5×10^6 cells, incubated for 4 days at 37° in 50 mm. plastic dishes in serum deficient ESOH 0.25% Ca medium, were infected at a multiplicity of exposure of 10 PFU per cell with pseudorabies virus. The infecting fluid used was medium removed from the cell sheet (0.5 ml./dish). After allowing 1 hr. at 37° for adsorption, the cell sheets were washed and 5 ml. of the 'used' medium added back to each plate. The cultures were reincubated at 37° and at selected times P.I. the total production of infectious virus from each of 2 cultures was estimated by plaque assay. The values from the duplicate plates were averaged and the titres (expressed as logarithms of total PFU per 10^6 cells initially plated) plotted against time P.I.

Figure 4.



increase in titre occurring, while from 10 hours till 24 hours post infection little increase in infectious titre was observed. The average yield of pseudorabies virus per cell was approximately 4 plaque forming units, a substantially lower yield (x100) than that obtained in the high serum system. In this system, cytopathic effect appeared at 8 or 9 hours post infection and was not typical of that normally associated with this virus since the cells did not all round up but tended to retain their characteristic shape and alter only in refractility or in a tendency to fuse together.

It would, therefore, seem that production of infectious virus does occur in cells deprived of serum on a time scale similar to that in actively growing cells, but that the overall virus yield is substantially diminished. The decrease observed may be attributable to either (a) virus multiplication at a reduced level occurring in every cell (due to the inactive state of the cells themselves or to deficiencies of essential growth components in the conditions provided) or (b) a very small percentage of the cells supporting viral replication at the normal level

(due to lowered cell viability in serum deficient conditions). No attempt has been made to resolve this question experimentally. It should perhaps be noted, that a situation similar to alternative (a) is observed following infection with herpes simplex in the absence of arginine (Tankersley, 1964).

(c) DNA Synthesis during Pseudorabies Virus Infection in a High Serum System.

The effect of infection of growing cultures of BHK21/13 cells with pseudorabies virus on the incorporation of [^{3}H] thymidine into trichloracetic acid insoluble material is illustrated in Fig. 5a. The bars on the histograms are estimates of the average incorporation of [^{3}H] label into acid insoluble material in coverslip cultures of uninfected or pseudorabies virus infected (at a multiplicity of 10 plaq forming units per cell) cells, exposed to the isotopically labelled compound for 1 hour in EC medium. The levels of thymidine incorporation into the uninfected cultures varied with time after mock-infection and this was probably attributable to only partially asynchronous DNA synthesis

Figure 5.

(a) Effect of Pseudorabies Virus Infection on DNA Synthesis in BHK21/13 Cells in a High Serum Tissue Culture System.

BHK21/13 cells, maintained in high serum medium on 13 mm. glass coverslips, were mock infected or infected with pseudorabies virus at a multiplicity of exposure of 10 PFU per cell. After adsorption for 1 hr. at 37°, the cultures were incubated at 37° in 2 ml. of EC medium. No attempt was made to synchronise infection. At selected times P.I. 10 μC of [^{3}H] thymidine (specific activity 1C/m-mole) was added and 1 hr. later the coverslips were removed, rinsed and fixed. The [^{3}H] label incorporated per coverslip in 1 hr. into trichloracetic acid insoluble product was plotted against time P.I. Each value shown is an average from 3 coverslips.

(b) Effect of Pseudorabies Virus Infection on DNA Synthesis in BHK21/13 Cells in a Serum Deficient Tissue Culture System.

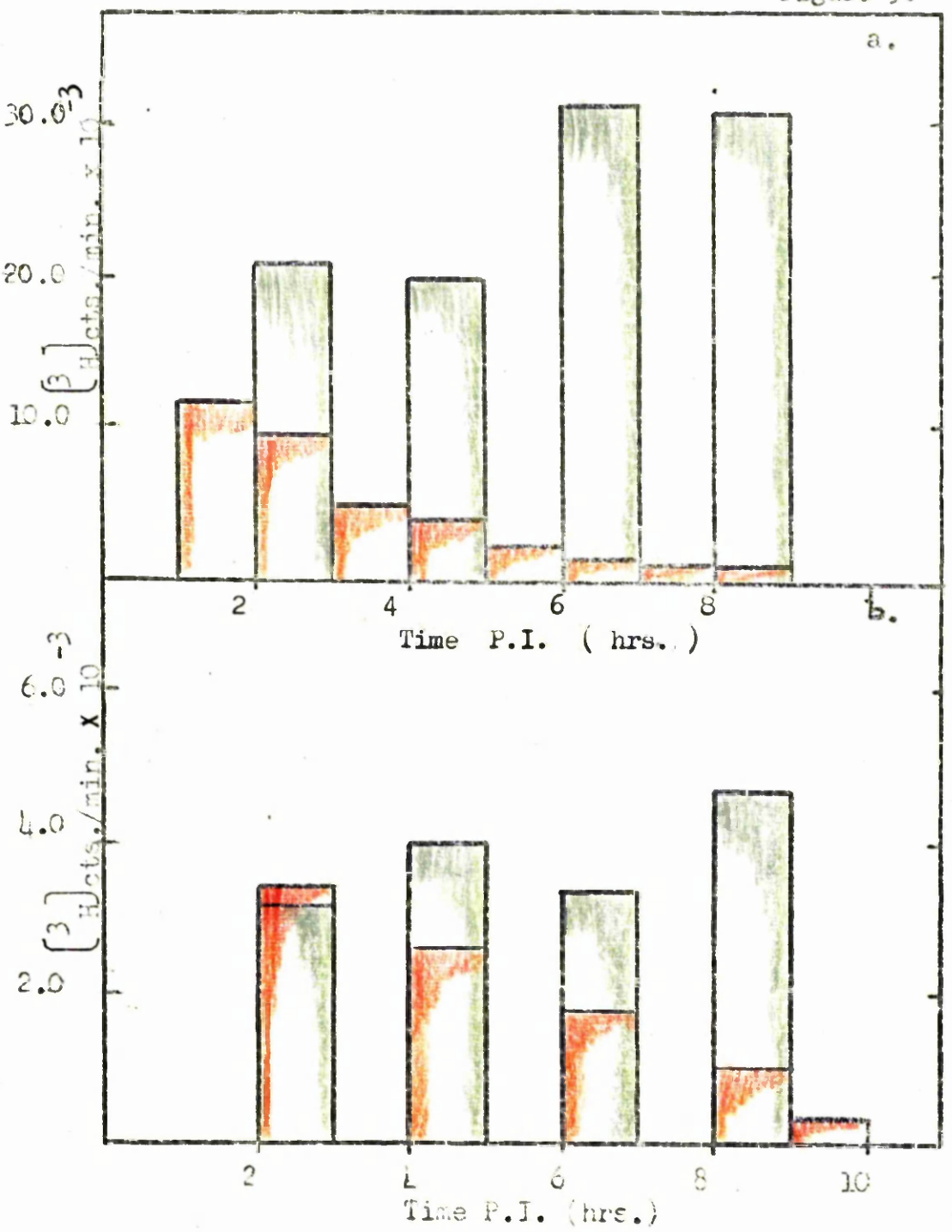
BHK21/13 cells, previously maintained for 4 days on 13 mm. coverslips in low serum ESOH medium, were mock infected or infected with pseudorabies virus at a multiplicity of exposure of 10 PFU per cell in 'used' medium. All subsequent procedures were as described in Section (a) except that the cultures were incubated in 2 ml. of 'used' medium.

□ [^{3}H] thymidine incorporation in non-infected BHK21/13 cells.

▭ [^{3}H] thymidine incorporation in virus infected BHK21/13 cells.

(cts./min./coverslip).

Figure 5.


a.
30.0
20.0
10.0
(^3H)cts./min. x 10^{-3}
2
4
6
8
b.
Time P.I. (hrs.)
6.0
4.0
2.0
(^3H)cts./min. x 10^{-3}
2
4
6
8
10
Time P.I. (hrs.)

in the cell population. Estimates of the [^{3}H] thymidine incorporated into infected cultures, made hourly from 1-9 hours post infection, provide evidence of a depression in incorporation as early as 1-2 hours post infection. After 5 hours of viral growth, the level of incorporation had gradually decreased to a constant level, 5%-10% of that in uninfected control cultures. These data cannot, however, be extrapolated to provide a similar comparison between the levels of total DNA synthesis in non-infected and virus infected cells. To obtain such a comparison, it would be necessary to arbritarily designate the proportions of viral and cellular DNA under synthesis at any particular time since thymidine comprises only 13% of the base residues in pseudorabies virus DNA (cf. 29% in cellular DNA). However, by assuming that pseudorabies virus shuts off cellular DNA synthesis very early in the infectious process and that by 2 hours post infection only viral DNA synthesis is occurring, certain conclusions can be drawn. It can, for instance, be inferred that viral DNA synthesis occurs mainly between 2-3 hours post infection when the overall level of DNA production

was marginally higher (115%) than that in corresponding non-infected cultures. By 4-5 hours post infection the level of synthesis is ~ 45% of that in the uninfected control cultures and from 5 hours onwards is only 10% of control values. This is, however, probably not an accurate reflection of the intracellular situation, for a limited degree of cellular DNA synthesis is probably occurring, especially early in infection.

(d) DNA Synthesis during Pseudorabies Virus Infection in a Low Serum System.

"Stationary" coverslip cultures of BHK21/13 cells, which had been exposed on plastic plates in used medium for 1 hour at 37° to 10 plaque forming units of pseudorabies virus per cell, were given 1 hour pulses of [^{3}H] thymidine from 2-3 hours, 4-5 hours, 6-7 hours, 8-9 hours post infection. Mock-infected cultures, pulsed at these times under identical conditions, were used as controls. Radioactive label incorporated into trichloracetic acid insoluble product was taken as a measure of thymidine incorporation into DNA. Reference to Fig. 5b shows that the levels of thymidine incorporation

in both non-infected and pseudorabies virus infected cells deprived of serum, differ from those observed in corresponding high serum cultures. Non-infected cells in the serum deficient system, for instance, incorporate into DNA in 1 hour only 10%-20% of the thymidine incorporated by their high serum counterparts. They also appear to form a more random, non-synchronised population. In the pseudorabies virus infected system, interpretation of the data reported is again, of course, complicated by the disparity in base composition between the viral and cellular DNA. However, values derived under the assumption that only viral DNA is synthesised after infection indicate that total DNA synthesis is again higher after, than before, viral infection but that levels in low serum infected cells are still 4 fold lower than those in corresponding high serum cells. The time scale of DNA synthesis is comparable in both systems since viral DNA synthesis in low serum cells also occurs maximally between 2 and 4 hours post infection and thereafter gradually diminishes. The overall pattern of comparison thus resembles that already described for infectious virus

production in actively growing and 'stationary' cultures, but it is also possible that the data reported here may be, at least in part, influenced by factors such as cell permeability for, or pool size of, thymidine.

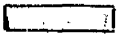
(e) General Protein Synthesis during Pseudorabies Virus Infection in a High Serum System.

Coverslip cultures of BHK21/13 cells, in plastic plates, which had been exposed in EC for 1 hour at 37° to 10 plaque forming units of pseudorabies virus per cell, were given 1 hour pulses of $[^3H]$ serine between 2 and 8 hours post infection. Uninfected cultures, pulsed from 2-3 hours, 4-5 hours and 6-7 hours, were used as controls. The radioactive label incorporated in this time into trichloracetic acid insoluble product was taken as a measure of the protein synthesis which had occurred. Reference to Fig. 6 shows the results, here represented as a histogram.. The level of $[^3H]$ serine incorporation into the uninfected cultures is practically unaltered throughout, indicating a constant rate of protein synthesis. In the pseudorabies virus infected cultures, the protein synthesis occurring had been reduced to 55% of the normal

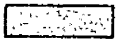
Figure 6.

Effect of Pseudorabies Virus Infection on Protein Synthesis in BHK21/13 Cells in a High Serum Tissue Culture System.

BHK21/13 cells, maintained in high serum medium on 13 mm. glass coverslips, were mock infected or infected with pseudorabies virus at a multiplicity of infection of 10 PFU per cell in 1 ml. EC medium in 50 mm. plastic plates. After adsorption for 1 hr. at 37°, a further 1 ml. of EC medium was added and the cultures incubated at 37°. No attempt was made to synchronise infection. At selected times P.I., 10 μC of [^{3}H] serine (specific activity 151 mC/m-mole) was added and 1 hr. later the coverslips were removed, rinsed and fixed by immersion in an acetic acid ethanol mixture. The [^{3}H] label incorporated per coverslip in 1 hr. into trichloracetic acid insoluble product was plotted against time P.I. Each value shown is an average of 3 coverslips.

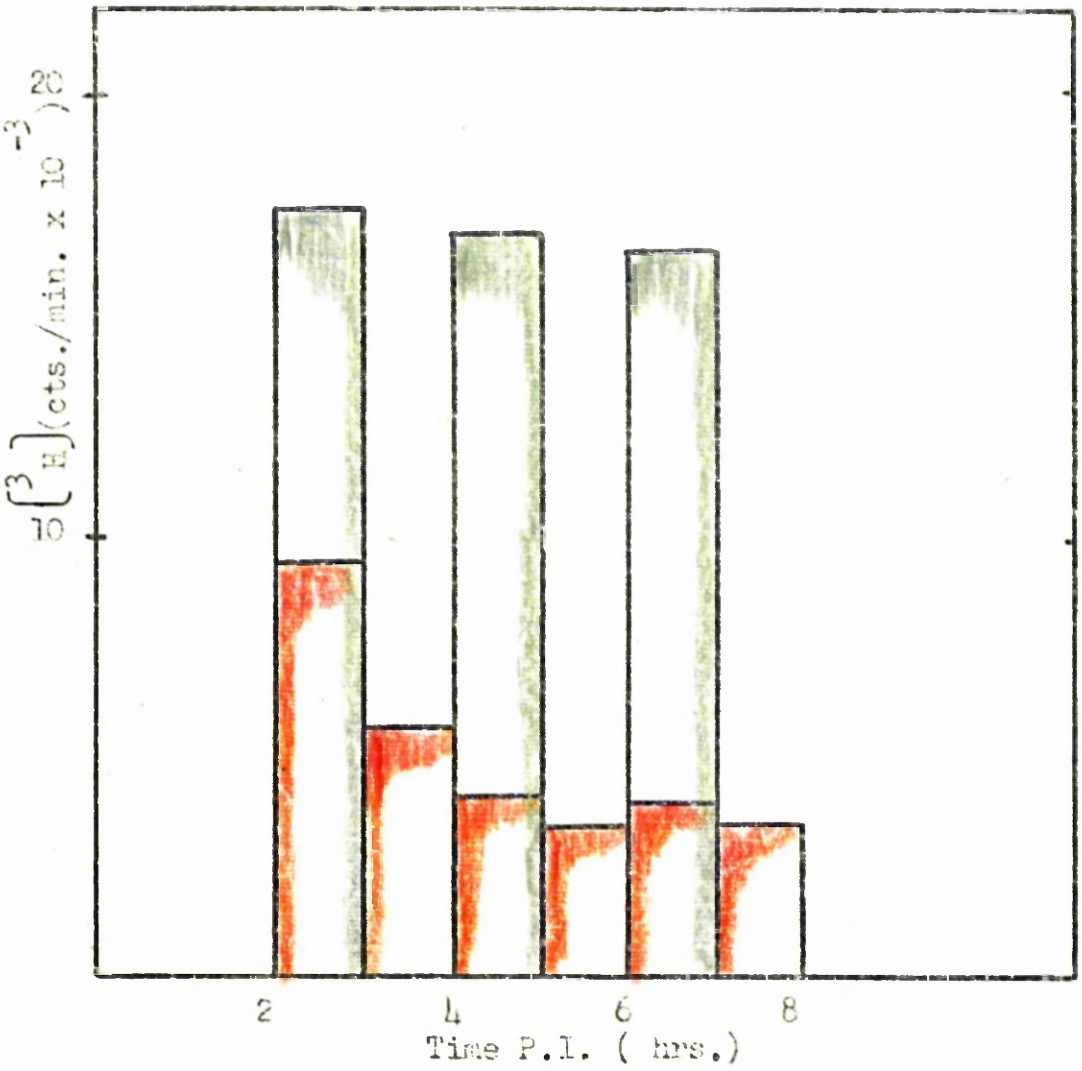


[^{3}H] serine incorporation by non-infected BHK21/13 cells (cts./min./coverslip)



[^{3}H] serine incorporation by viral infected BHK21/13 cells (cts./min./coverslip)

Figure 6.



by 2-3 hours post infection, to 20% by 3-4 hours post infection and to 14% by 4-5 hours post infection. The incorporation then remained at this value at least up to 8 hours post infection. Thus, like most lytic viruses, pseudorabies immediately on infection affects the rate of protein synthesis in the cell, with the result that the rate is reduced after 4 hours post infection to 15% of that in uninfected cells.

(f) Incorporation of Arginine and Lysine during Pseudorabies Virus Infection in a High Serum System.

The effect of pseudorabies virus infection of actively growing cultures of BHK21/13 cells on the simultaneous incorporation of the amino acids arginine and lysine into trichloracetic acid insoluble material (500 μg. of protein as measured by the method of Lowry et al. (1951))is shown in Figure 7. Each point represents the ratio of the mμ-moles $\left[^{14}C\right]$ arginine incorporated to mμ-moles $\left[^{3}H\right]$ lysine incorporated in 15 minute pulses into acid insoluble material at various times after pseudorabies virus infection. Cultures, mock-infected with EC, were used as controls. Ornithine was added with the labelled amino acids to prevent any interconversion of the labels

Figure 7.

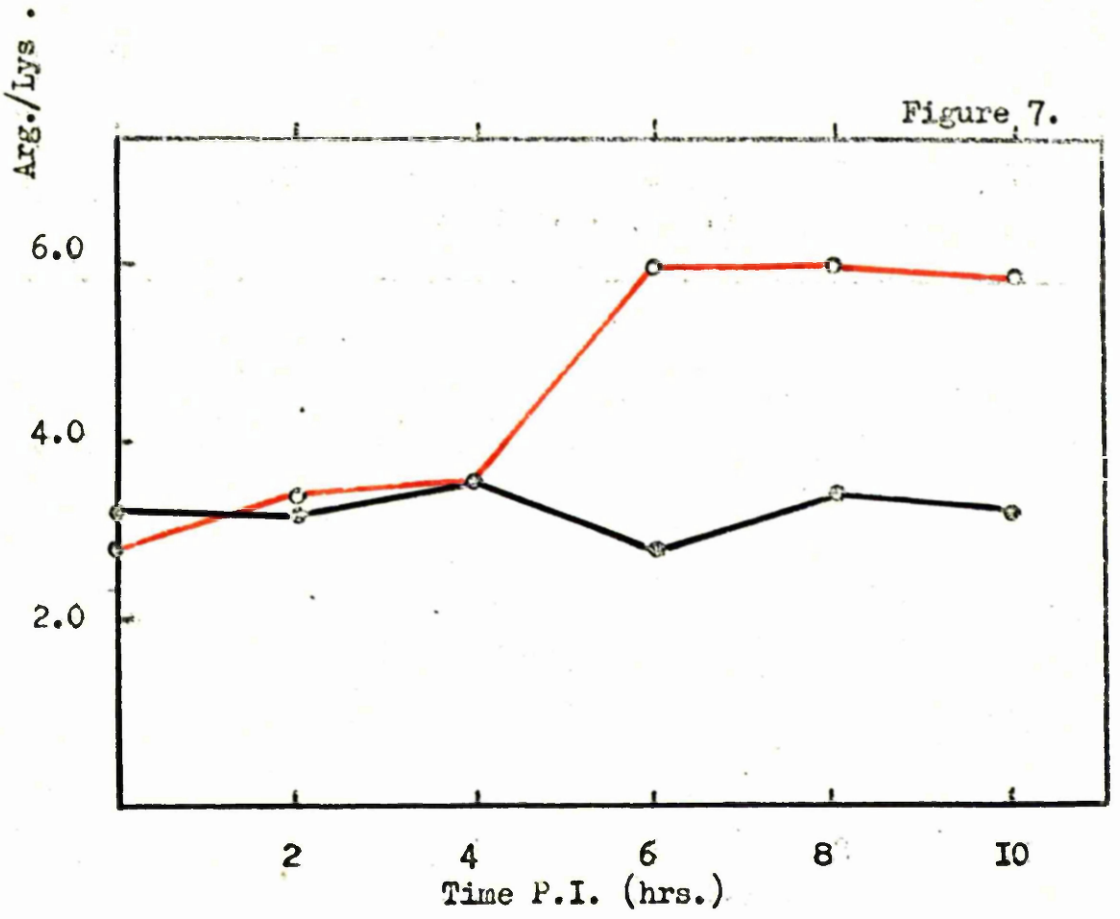
Effect of Pseudorabies Virus Infection on the Relative Incorporation of Arginine and Lysine into Protein in a High Serum Tissue Culture System.

BHK21/13 cells, maintained in baby bottle cultures in high serum medium, were mock infected or infected with pseudorabies virus in 0.5 ml. of EC medium at a multiplicity of exposure of 20 PFU per cell. After adsorption for 1 hr. at 37°, 10 ml. of EC medium was added and the cultures reincubated. No attempt was made to synchronise infection. At selected times P.I., 2 mock infected and 2 virus infected cultures were pulsed for 15 mins. in 1 ml. of EC containing 2.5 μmoles of ornithine per ml. and differentially labelled with a mixture of $[^{14}C]$ arginine and $[^{3}H]$ lysine. The specific activity of $[^{14}C]$ arginine was 5.5 mC/m-mole while that of $[^{3}H]$ lysine was 10 mC/m-mole. The pulse period was immediately followed by a 10 min. chase in EC, containing 20 x the normal concentrations of arginine and lysine hydrochlorides. After these cultures had been harvested and the cells disrupted, estimates were made of the $[^{3}H]$ and $[^{14}C]$ disintegrations from acid insoluble material (500 μg. of protein) and the incorporation ratios calculated. The values plotted against time P.I. are each the average of 2 estimations.

——— Arg./lys. in non-infected BHK21/13 cells
mμmoles arg. incorp./mμmoles lys. incorp.

——— Arg./lys. in virus-infected BHK21/13 cells
mμmoles arg. incorp./mμmoles lys. incorp.

Figure 7.



by endogenous pathways. Cultures were also subjected to a 10 minute chase period to ensure that the label was not present in the form of aminoacyl-tRNA. The ratios might, therefore, be regarded as estimates of the relative amounts of arginine and lysine present in the proteins under synthesis. The ratio of the arginine incorporated to the lysine incorporated (arg./lys.) into protein in the uninfected cultures remains relatively constant at a value of around 3.0 at all times after treatment. Up to 4 hours post infection, the same ratio in the protein of the pseudorabies virus infected cultures shows no marked deviation from the normal pattern. However, between 4 and 6 hours post infection, the ratio altered to a value in the region of 6.0. This is only a 2 fold increase but each of the points shown is an average of two almost identical values. The simplest explanation is, that between 4 and 6 hours post infection, the infected cells start to synthesise proteins which are either richer in arginine or deficient in lysine compared to those being synthesised in the control cells. The results could also possibly be explained on the basis of differential alterations in the pool sizes of arginine

and lysine. This hypothesis has not been disproved experimentally, but the previous explanation is favoured, especially since the alteration in ratio occurred at the commencement of maximal production of viral coat proteins, the stage at which the multiplication of herpes simplex virus is blocked in arginine deficient culture conditions (Becker, Olshevsky & Levitt, 1967).

(g) Incorporation of Arginine, Lysine and Valine during Pseudorabies Virus Infection in a Low Serum System.

An essentially similar result was obtained using cultures of BHK21/13 cells in which metabolism had been depressed by deprivation of serum. In this system, the incorporation ratio (arg./lys.) in uninfected cells was again relatively constant, while in pseudorabies virus infected cells the value, after remaining constant up to 5 hours post infection, increased two-fold between 5 and 6 hours post infection. This value was maintained at least till 10 hours post infection.

The alteration of the ratio of arginine incorporation to lysine incorporation resulting from pseudorabies virus infection (as described above) could obviously be due

either to increased arginine incorporation or to decreased lysine incorporation. Therefore, a further amino acid, valine, was chosen as marker. Comparisons valine and $[^3H]$ arginine, ultaneous incorporation of $[^{14}C]$ valine and $[^3H]$ arginine, $[^{14}C]$ lysine and $[^3H]$ valine and, as control, $[^{14}C]$ lysine and $[^3H]$ arginine. It is obviously necessary to assume that $[^{14}C]$ valine and $[^3H]$ valine are identically metabolised by the cells - a necessary assumption in all radioactive double labelling experiments. The techniques used in these experiments were essentially identical to those outlined above. Metabolically inactive cells in serum deficient medium, either mock-infected or pseudorabies virus infected were used.

The incorporation ratios (arg./lys.), (lys./val.) and (arg./val.) determined in mock-infected cultures remained relatively constant at all times during the experiment. Average values for the 3 ratios were, therefore, calculated for use as correction factors. In the data presented in Fig. 8, the values for the incorporation ratios from infected cells are thus referred to a control value of unity.

The pattern obtained at specified times after viral

Figure 8.

Effect of Pseudorabies Virus Infection on the Relative Incorporation of Arginine, Lysine and Valine into Protein in a Serum Deficient System.

BHK21/13 cells, previously maintained for 4 days in a low serum medium, were mock infected or infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell (in 0.5 ml. of used medium) After adsorption for 1 hr. at 37° a further 1.5 ml. of medium was added and the cultures reincubated. At selected times P.I., 2 control and 2 infected cultures were pulsed for 15 mins. in used medium (2.5 mM-ornithine), with a differentially labelled mixture of $[^3H]$ arginine (15 mC/m-mole) and $[^{14}C]$ lysine (0.7 mC/m-mole). $[^3H]$ arginine and $[^{14}C]$ valine (1.25 mC/m-mole) or $[^{14}C]$ lysine and $[^3H]$ valine (7.5 mC/m-mole). The pulse period was immediately followed by a 10 min. chase in EC, containing 20 x the normal concentrations of the amino acids mentioned. After these cultures had been harvested and the cells disrupted, estimates were made of the $[^3H]$ and $[^{14}C]$ disintegrations from trichloracetic acid insoluble material (200 µg. of protein). The values plotted against time P.I. are each an average of 2 separate estimations and are corrected to a control value of unity.

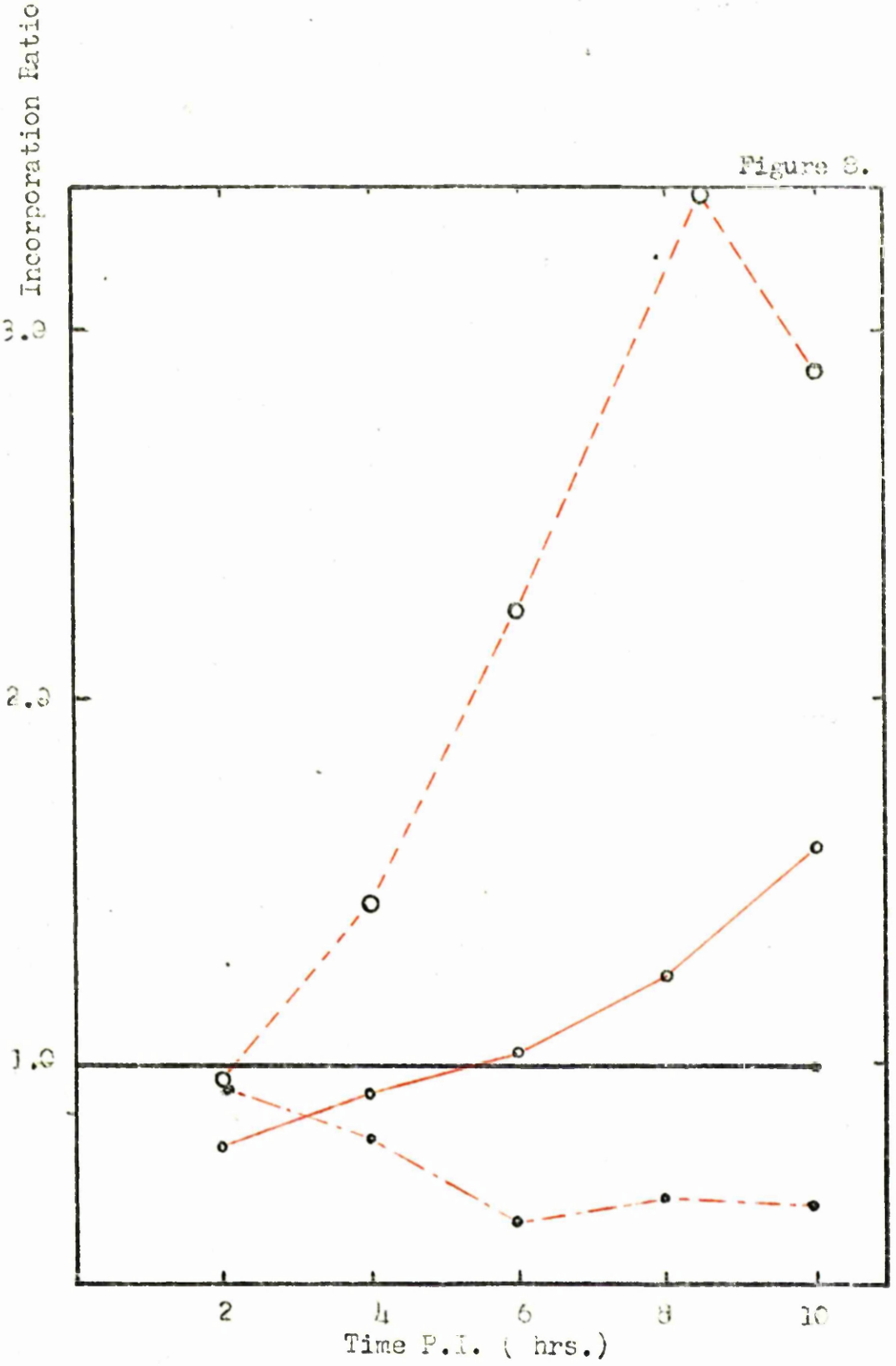
——— Arg./lys.; arg./val.; and lys./val. in non-infected cells.

– – – Arg./lys. in virus infected cells.

——— Arg./val. in virus infected cells.

– – – – Lys./val. in virus infected cells.

Figure 8.



infection for (arg. /lys.) differed slightly from that previously described. In this case, the increase observed in the ratio was not a single step process but continued for some hours. From 2 hours post infection onward, the value steadily increased until at approximately 8 hours post infection it eventually reached a constant value about 3 times that in uninfected cells. The (arg. /val.) ratio also slowly increased with time after pseudorabies virus infection and eventually reached at 10 hours post infection, a marginally higher value (1.5 times) than that of the constant control. A decrease of similar magnitude was also observed at 10 hours post infection in the value of (lys. /val.) ratio.

The experimentally observed alterations in the incorporation ratios of arginine to valine and lysine to valine appear, at first sight, insufficient to account for the three-fold increase in the incorporation ratio of arginine to lysine. However, direct comparison of the (arg. /lys.) values observed with those calculated from the (val. /lys.) and (arg. /val.) values can be seen (Fig. 9) to agree reasonably well (arg. /val. x val. /lys.). The calculated

Figure 9.

Effect of Pseudorabies Virus Infection on the Relative Incorporation of Arginine, Lysine and Valine in a Serum Deficient System.

The experimental details are given in Fig. 8.

Arg./lys. = mμmoles arg. incorp./mμmoles lys. incorp.

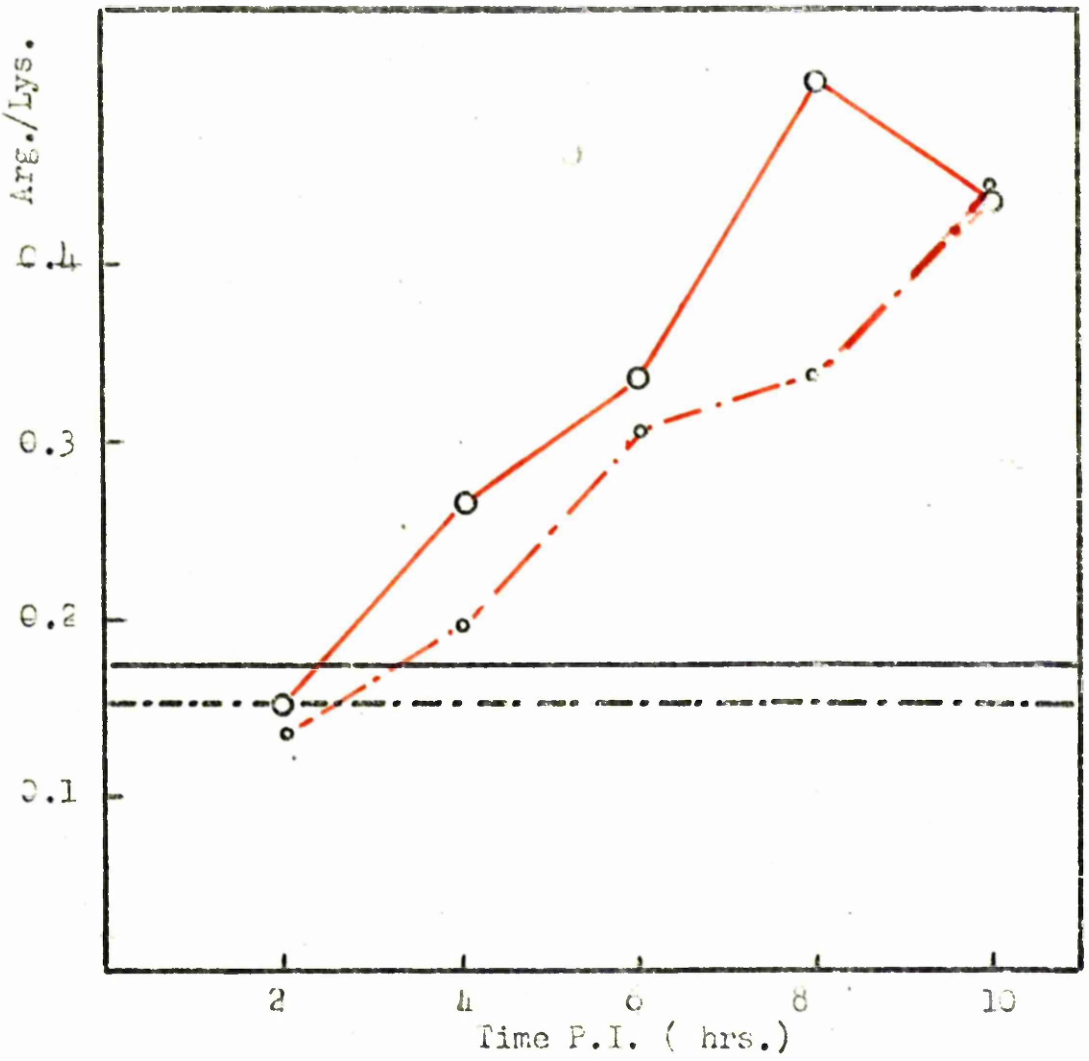
_____ Average arg./lys. in non-infected BHK21/13 cells as experimentally determined.

_ . _ . _ Average arg./lys. in non-infected BHK21/13 cells as calculated (arg./val. x val./lys.)

_____ Arg./lys. in virus infected BHK21/13 cells as experimentally determined.

_ . _ . _ Arg./lys. in virus infected BHK21/13 cells as calculated (arg./val. x val./lys.).

Figure 9.



values give consistently lower values than those observed experimentally. It seems reasonable to deduce, therefore, that the change in protein synthesis reflected in the (arg./lys.) values is due to a composite effect, dependent both on an increase in arginine incorporation and a decrease in the lysine incorporation into proteins synthesised after pseudorabies virus infection. It is, however, difficult to quantitate the effect precisely since the level of incorporation of the valine marker may also be higher in infected than in uninfected cells.

Therefore, although the results presented can possibly be explained in terms of altered amino acid pools, increased cell permeability, etc., and do not provide rigorous proof of an alteration in the balance of protein synthesis following pseudorabies infection, it seems reasonable to conclude that the proteins synthesised in pseudorabies virus infected cells, from 4 hours post infection onwards, are richer in arginine and poorer in lysine than those in the non-infected controls. Indeed, the most reasonable explanation would be that the change in the value of (arg./lys.) during infection coincides with

the onset of synthesis of viral coat proteins and is attributable to copious expression of a DNA of high G+C base composition in polypeptide synthesis.

In short, it has been found that:

(a) The pseudorabies virus strain used had a lytic cycle (of 8 hours) in BHK21/13 cells, identical with that described by Kaplan & Vatter (1959) in RK cells. The viral yield in a high serum system also corresponded to that from RK cells. In a serum deficient system, the yield was substantially reduced.

(b) In common with the Kaplan strain of pseudorabies virus and other lytic viruses, the strain isolated caused depression of host DNA and protein synthesis from early in infection.

(c) The proteins synthesised from 4 hours post infection onward contained a higher proportion of arginine and a lower proportion of lysine than those from uninfected cells.

In the biochemical investigations of low molecular weight RNAs to be described in the following three sections, uninfected cells were compared to cells harvested at least

6 hours after infection with pseudorabies virus. For reasons already discussed in the introductory section (see p.65), the conditions at this time should be optimal for the detection of any virus induced alterations in the composition of aminoacyl-tRNA populations.

3. THE METABOLISM OF PULSE-LABELLED LOW MOLECULAR WEIGHT RNAs IN NON-INFECTED AND PSEUDORABIES VIRUS INFECTED BHK21/13 CELLS

Sephadex-G100 column chromatography is known to separate low molecular weight RNA from species of substantially higher molecular weight such as ribosomal or mRNA and also to resolve the low molecular weight RNA into its 2 main components - 4s and 5s (Rosset, Monier & Julien, 1964). A typical elution pattern of a multi-component RNA preparation, obtained after routine chromatographic procedures, is illustrated in Fig. 10. By comparison with published elution profiles, the resultant peaks of U. V. absorbing material centred at fraction 36, at fraction 46 and at fraction 60, subsequently termed A, B and C, can be identified as respectively ribosomal + mRNA, 5s RNA and 4s RNA. Verification of the identification of peak C as 4s RNA was obtained by chromatographing a preparation of $[^{14}C]$ alanyl-tRNA on an identical Sephadex column when, accounting for slight differences

Figure 10.

Elution Pattern Obtained by Gel Filtration of 'Cytoplasmic' RNA on Sephadex-G100.

An unlabelled preparation of RNA from the cytoplasmic fraction of non-infected BHK21/13 cells was chromatographed at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. 50 mM - sodium acetate buffer, pH 5.5 was used for elution and 1 ml. fractions were collected. Each fraction was assayed for extinction at 260 mμ.

----- E_{260}

Figure 10.

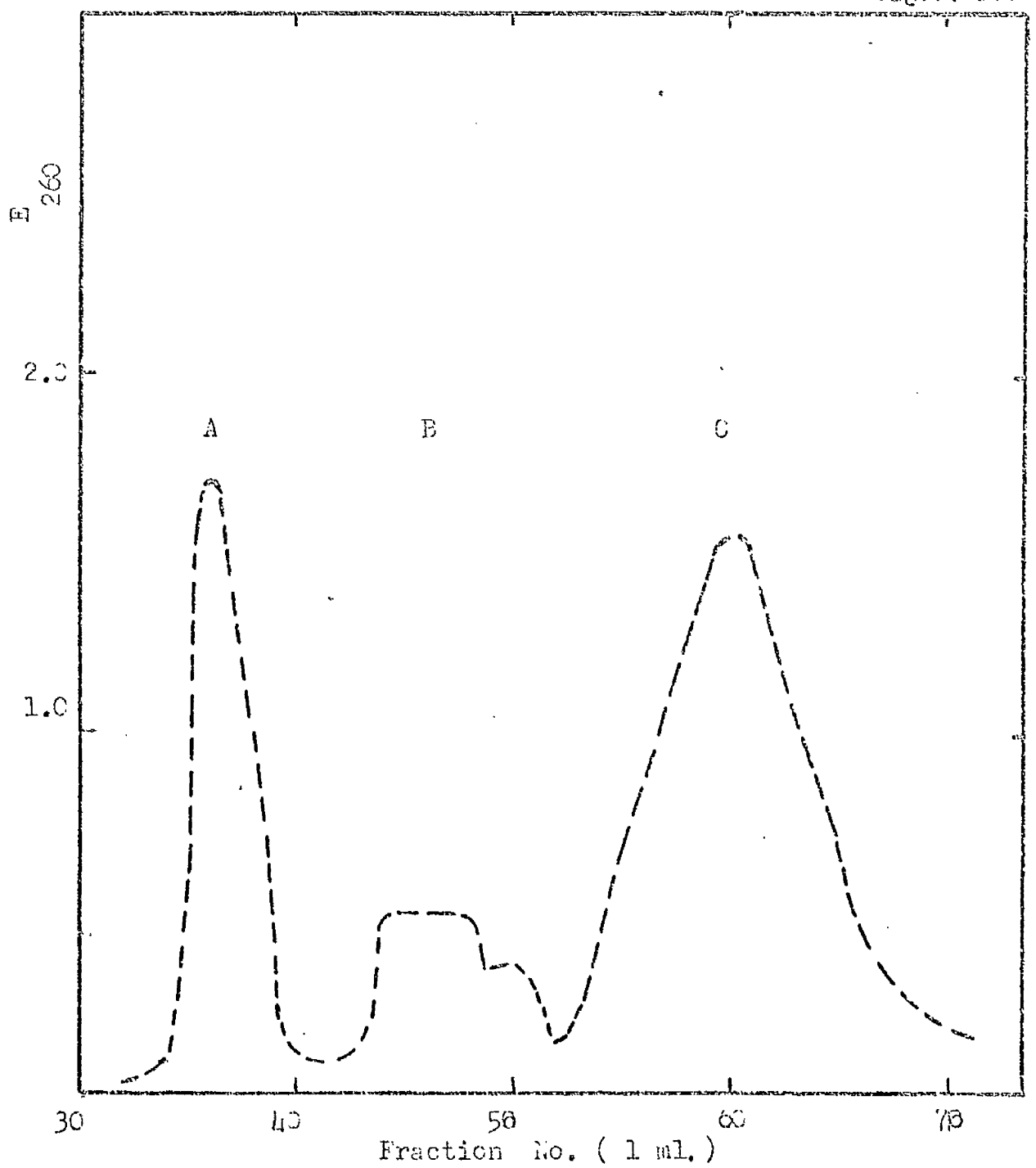


Figure 11.

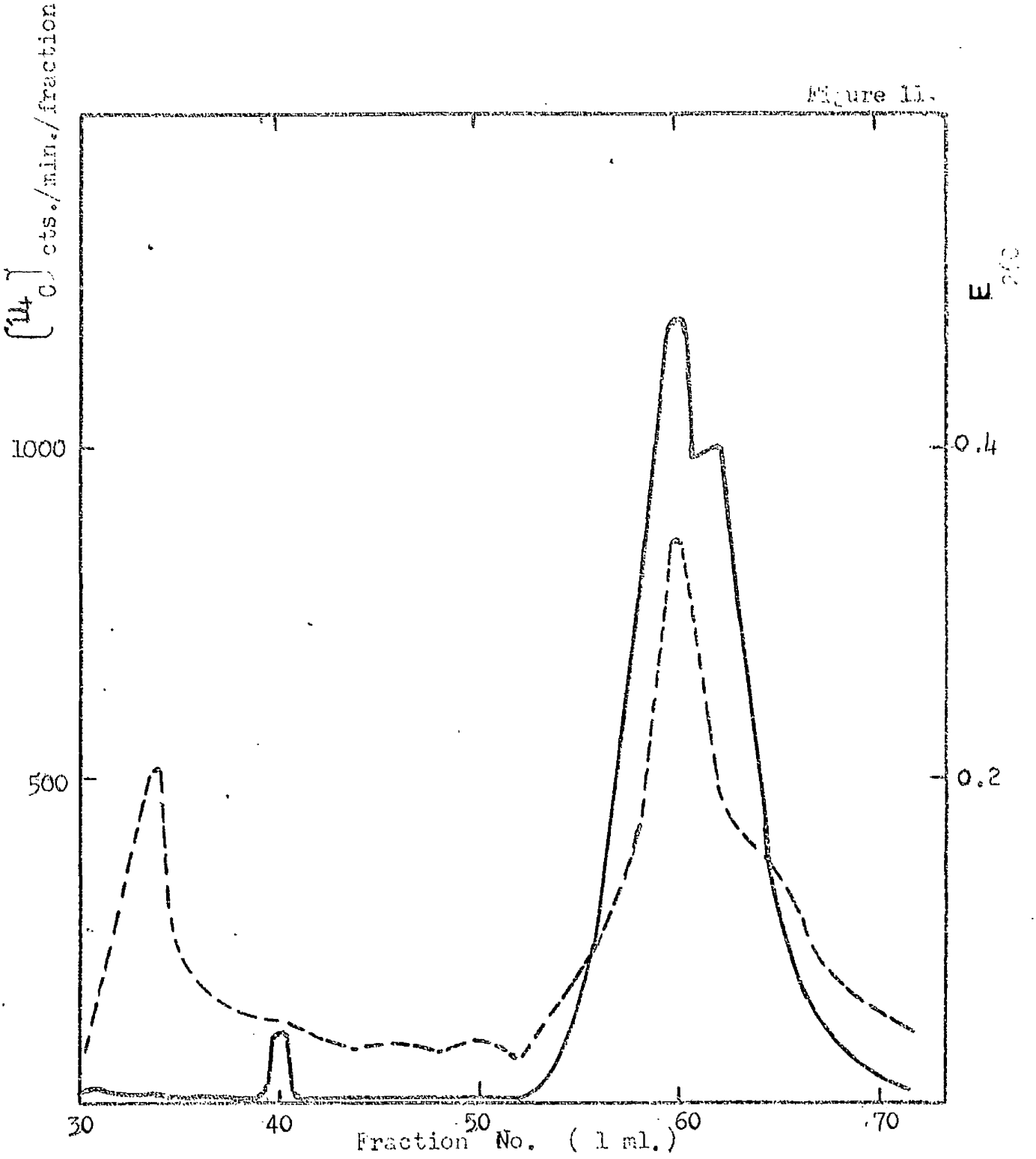
Elution Pattern Obtained by Gel Filtration of Aminoacyl-tRNA on Sephadex-G100.

A preparation of aminoacyl-tRNA labelled with [^{14}C] alanine was chromatographed at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. 50 mM - sodium acetate buffer, pH 5.5, was used for elution and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for trichloracetic acid insoluble radioactivity.

----- E_{260}

——— [^{14}C] cts./min./fraction.

Figure 11.


$[^{14}C]$ cts./min./fraction
1000
500
E 280
0.4
0.2
30
40
50
60
70
Fraction No. (1 ml.)

in reproducibility between columns, all the radioactive label and, therefore, all the aminoacyl-tRNA was eluted in position C (Fig. 11).

This fractionation was used to investigate the RNA components extracted from nuclear and cytoplasmic fractions of mixed populations of non-infected and 6.5 hour pseudorabies virus infected BHK21/13 cells, the RNA from the uninfected cells being pulse-labelled for 30 minutes with uridine - $[^{14}C]$ (G) and that from virus infected cells with $[^{3}H]$ uridine (-5-T).

In the nuclear fraction of uninfected and pseudorabies infected cells (Fig. 12), all the RNA detected, both pre-existant as measured by extinction at 260 mμ and newly synthesised, as detected by the incorporation of $[^{14}C]$ or $[^{3}H]$ label, eluted with the void volume of the column (peak A). It, therefore, appears to be of high molecular weight. The small peak of absorbance at 260 mμ appearing in position C is completely attributable to added unlabelled 4s marker RNA. Similarly, in the G100 elution pattern of nuclear RNA from differentially labelled uninfected and pseudorabies virus infected cultures which had been incubated, after labelling, for a further hour in uridine rich growth medium, only a single major peak containing both U.V. absorbing material

Figure 12.

Comparison of Pulse-labelled (30 min.) 'Nuclear' RNA of Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Gel Filtration on Sephadex-G100.

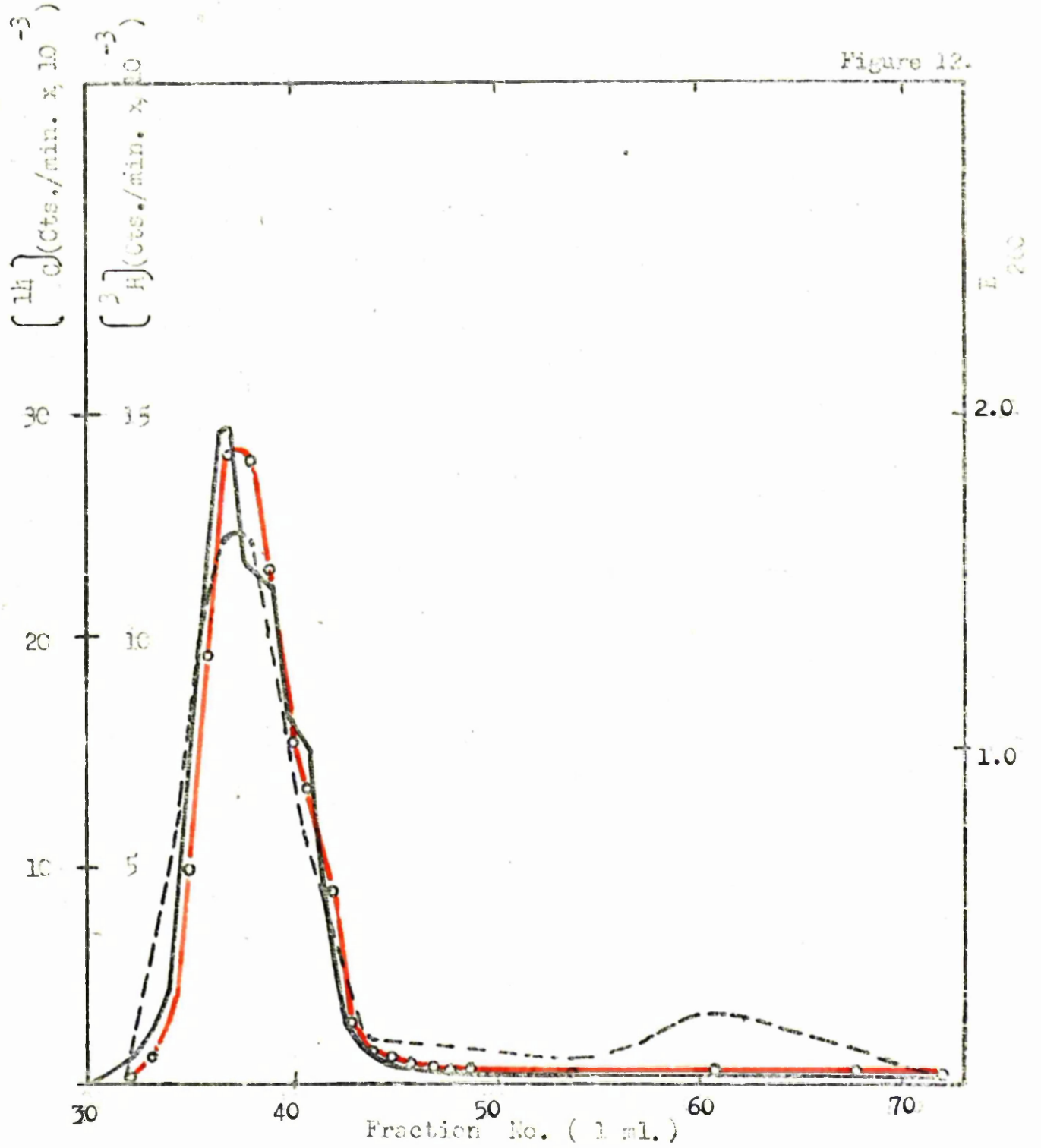
Roller bottle cultures of actively growing BHK21/13 cells, mock infected or infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell, were pulse-labelled from 6 to 6.5 hrs. P.I. with $[^{14}C]$ uridine (G) and $[^{3}H]$ uridine (-5-T) respectively. (500 μC of $[^{3}H]$ uridine of specific activity 8.75 C/m-mole or 100 μC of $[^{14}C]$ uridine of specific activity 400 mC/m-mole were provided per roller bottle culture). The cells were immediately harvested and pooled. 'Nuclear' RNA, extracted from a 1:1 mixture of these cells, was fractionated at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. Unlabelled 4s RNA was added as marker. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for total radioactivity associated with each isotope.

---- E_{260}

——— $[^{3}H]$ cts./min./fraction.

——— $[^{14}C]$ cts./min./fraction

Figure 12.


[14C](Cts./min. x 10-3)
[3H](Cts./min. x 10-3)
E280
30
20
10
2.0
1.0
30
40
50
60
70
Fraction No. (1 ml.)

and radioactivity was present. There is, therefore, no evidence in the mixed nuclear RNA populations examined of any newly synthesised low molecular weight RNA or of any such species being subsequently produced by, say, degradation of a higher molecular weight species. Such species might be revealed by using a stronger extraction procedure than the SDS - cold phenol technique employed (Knight & Darnell, 1967).

Reference to Fig. 13 illustrates the G100 elution pattern of RNA extracted by the cold phenol method from the cytoplasmic fraction of the radioactively pulse-labelled uninfected and pseudorabies virus infected cells, the nuclear fraction of which has already been examined. Examination of the regions of maximum absorbance at 260 mμ show that, as expected, the cytoplasm of a mixture of uninfected and virus infected BHK21/13 cells contains 3 'types' of RNA eluting at positions A, B and C, there being a high proportion of ribosomal + m species, a lower but still substantial proportion of 4s RNA (21%) and a small but identifiable proportion of 5s RNA (7% total).

When newly synthesised RNA from the cytoplasmic fraction of uninfected cells - as identified by the $[^{14}C]$ label - was examined, the radioactivity derived from the $[^{14}C]$ uridine was

Figure 13.

Comparison of Pulse-labelled (30 min.) 'Cytoplasmic' RNA of Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Gel Filtration on Sephadex-G100.

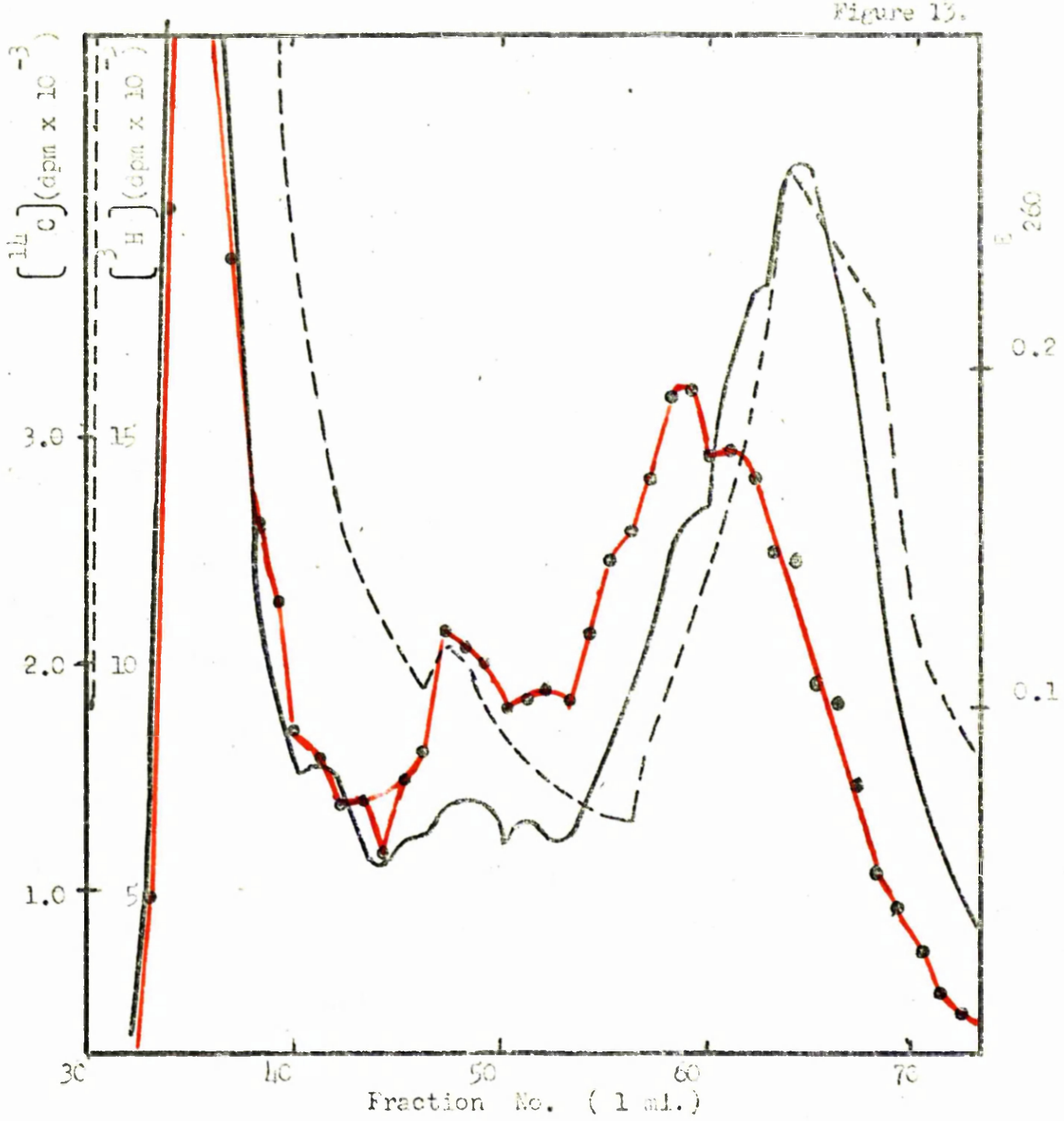
Roller bottle cultures of actively growing BHK21/13 cells were mock infected or infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell and pulse-labelled from 6 to 6.5 hrs. P.I. with [^{14}C] uridine (G) and [^{3}H] uridine (-5-T) respectively. (500 μC of [^{3}H] uridine of specific activity 8.75 C/m-mole or 100 μC of [^{14}C] uridine of specific activity 400 mC/m-mole were provided per roller bottle culture). The cells were immediately harvested and pooled. 'Cytoplasmic' RNA, extracted from a 1:1 mixture of these cells, was fractionated at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for total radioactivity associated with each isotope.

---- E_{260}

——— [^{3}H] disintegrations/min./fraction.

——— [^{14}C] disintegrations/min./fraction.

Figure 13.


[14C](dpm x 10-3)
[3H](dpm x 10-3)
E 260
3.0
2.0
1.0
15
10
5
0.2
0.1
30
40
50
60
70
Fraction No. (1 ml.)

found in positions corresponding to ribosomal, 5s and 4s RNA. The radioactivity associated with these species could be correlated positionally almost exactly with the corresponding optical density peaks. Only as regards the 5s peak was any relative displacement observed. The distribution of the label among these peaks was slightly different to that observed by measurement of U. V. absorbance since only 38% of the labelled material was located in the ribosomal peak, 13% in the 5s peak and around 50% in the 4s peak.

On examination of the elution profile obtained in the above chromatographic experiment for simultaneously prepared $[^3H]$ labelled RNA from pseudorabies virus infected cell cytoplasm, a more complicated pattern emerges. 3 main peaks of radioactive material are again present. Only 2 of these, however, the ribosomal and the 5s RNA components can be superimposed on the corresponding U. V. absorbing peaks. The 3rd component containing $[^3H]$ label elutes in a position between the 5 and 4s species, at least 6 fractions earlier than the 4s RNA absorbance peak, and has a relatively high specific activity. Material eluting in this position will subsequently be termed $4\frac{1}{2}$s material. Similar control and infected patterns were obtained when

chromatography was carried out at pH 5.5 in 50mM-ammonium acetate buffer. The relative proportions of the 3 species is different to that in uninfected cells, 26% being ribosomal + mRNA, 22% 5s RNA and 52% 4s RNA. This alteration in the distribution of labelled material among the 3 peaks will be more fully investigated later and discussion of the subject is, therefore, deferred till then.

The peak of $\{^3H\}$ activity occurring in the $4\frac{1}{2}$s position in the 30 minutes labelling experiment outlined may represent the accumulation, in pseudorabies virus infected cells, of an intermediate in the process leading to the formation of 4s RNA (Burdon, Martin & Lal, 1967). Such an intermediate is assumed to be less effectively retained on columns of Sephadex-G100 because it occupies a larger molecular volume than the 'mature' species. The following experiments were carried out to determine the applicability of this hypothesis and to relate the phenomenon to the general picture of virus growth.

To investigate the effect initiated by pseudorabies virus infection on the pattern of low molecular weight RNA synthesis, infected cell cultures were pulse-labelled for 30 minutes with $\{^3H\}$ uridine at various times post infection and the RNA extracted

from the cytoplasm then examined by G100 column chromatography. The resultant elution profiles are shown in Fig. 14. Several obvious differences exist but, at this stage, only the elution position of the 4s peak will be considered. Comparisons between columns and, therefore, between preparations, must be made by reference to the standard elution profile of U. V. absorbing material since there was no (^{14}C) uridine-labelled uninfected RNA as internal marker. Previous examination of the distribution of (^{3}H) label and absorbance at 260 mμ in pulse-labelled 'cytoplasmic' RNA from uninfected cells had confirmed that in the 4s region the radioactivity derived from (^{3}H) uridine appears in a relatively concise band which can be exactly correlated positionally with the optical density peak.

By 2-2.5 hours post infection, the elution profile of the (^{3}H) labelled RNA in the 4s region has altered when compared to that of uninfected cells. The labelled material now elutes from G100 slightly earlier than before and is dispersed over a larger elution volume - the radioactivity, in addition to eluting with the E_{260} peak, showing a distinct leading edge. In gel filtration profiles of RNA labelled between 4 - 4.5 hours post infection, 6 - 6.5 hours post infection and 8 - 8.5 hours post infection, the 3rd peak

Figure 14.

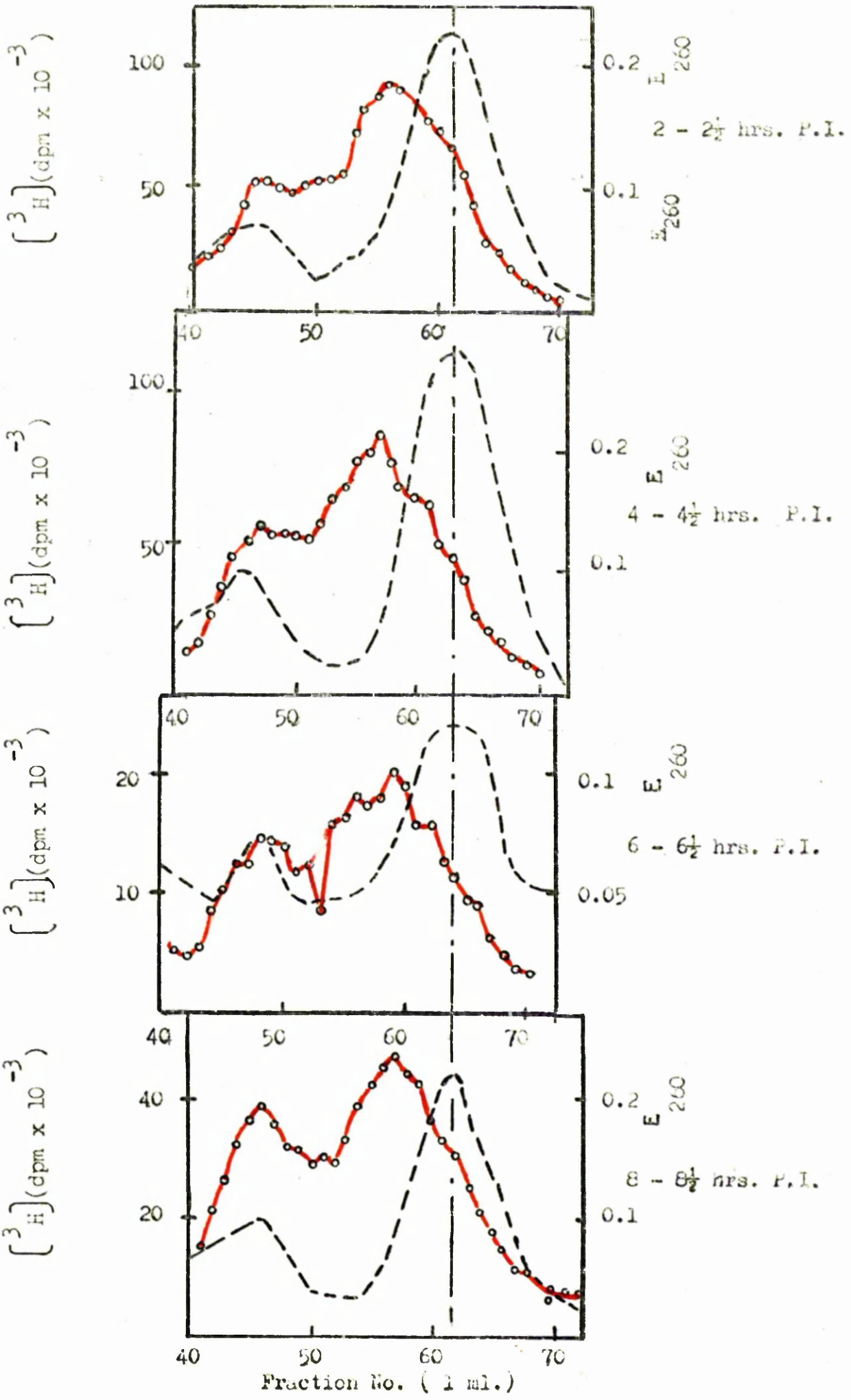
Comparison by Gel Filtration on Sephadex-G100 of Pulse-labelled (30 min.) 'Cytoplasmic' RNA from BHK21/13 Cells infected for Different Time Intervals with Pseudorabies Virus.

Roller bottle cultures of actively growing BHK21/13 cells were infected at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus and each pulse-labelled with 750 μC of [^{3}H] uridine (-5-T) (specific activity 8.6 C/m-mole from i) 2 - $2\frac{1}{2}$ hrs. P.I. ii) 4 - $4\frac{1}{2}$ hrs. P.I. iii) 6 - $6\frac{1}{2}$ hrs. P.I. and iv) 8 - $8\frac{1}{2}$ hrs. P.I. The cells were immediately harvested and the 'cytoplasmic' RNA extracted from them fractionated, at room temperature, on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0, was used as eluant and the 1 ml. fractions collected were immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for total [^{3}H] radioactivity.

---- E_{260}

——— [^{3}H] disintegrations/min./fraction.

Figure 14.


[3H](dpm x 10-3)
100
50
0.2
0.1
E260
2 - 2½ hrs. P.I.
40
50
60
70
100
50
0.2
0.1
E260
4 - 4½ hrs. P.I.
40
50
60
70
20
10
0.1
0.05
E260
6 - 6½ hrs. P.I.
40
50
60
70
40
20
0.2
0.1
E260
8 - 8½ hrs. P.I.
40
50
60
70
Fraction No. (1 ml.)

of radioactivity elutes entirely in the $4\frac{1}{2}$s position and not in the 4s position (as defined by the region of U. V. absorbance). This confirms the result obtained in the double labelling experiment already described. It would, therefore, seem that from early in pseudorabies virus infection a previously unobserved RNA component ($4\frac{1}{2}$s) gradually accumulates in the cell cytoplasm. Since the accumulation, which has reached detectable levels by 2 hours post infection, can be thereafter correlated with a decreasing ability for rapid 4s RNA synthesis, the RNA is perhaps a normally transitory intermediate in cellular 4s RNA synthesis.

When an identical RNA extraction and analysis procedure was carried out with uninfected BHK21/13 cells using a decreased incubation period of 10 minutes, less $\left[^3H\right]$ radioactivity was present in all fractions, especially those corresponding to ribosomal RNA (see Fig. 15). 4s RNA synthesis must therefore occur more rapidly than ribosomal. The $\left[^3H\right]$ radioactivity was, throughout, fairly closely associated with the main RNA species although the peak of 4s RNA material showed perhaps a slight indication, in the diffuse leading edge, of the presence of RNA species less effectively retained by columns of Sephadex-G100. However, in general, the results indicate that the 4s RNA components

Figure 15.

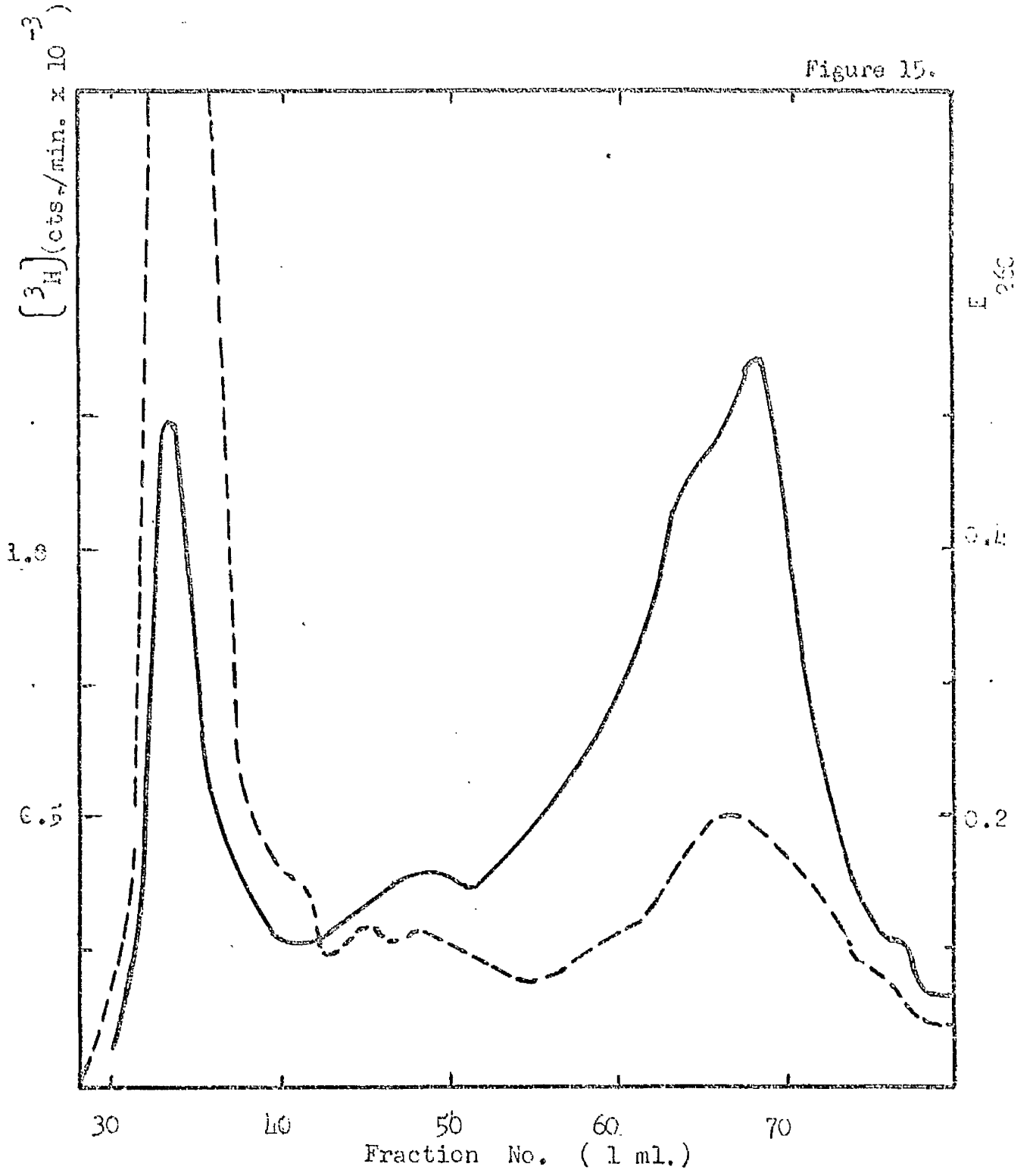
Elution Pattern obtained by Gel Filtration on Sephadex-G100 of Pulse-labelled (10 min.) 'Cytoplasmic' RNA from Non-infected BHK21/13 Cells.

RNA was prepared from the 'cytoplasmic' fraction of actively growing non-infected BHK21/13 cells which had been pulse-labelled with [^{3}H] uridine (-5-T) (500 μC of specific activity 8.75 C/m-mole per roller bottle culture) for 10 mins. immediately before harvesting. The RNA, preparation so obtained, was fractionated at room temperature on a 1.5 x 80 cm. column of Sephadex-G100 using SSC as eluant (pH 7.0). 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for total [^{3}H] radioactivity.

--- E_{260}

——— [^{3}H] cts./min./fraction.

Figure 15.



synthesised in 10 minutes by uninfected cells were predominantly in the completed form. If $4\frac{1}{2}$s RNA is indeed an intermediate in the synthesis of 4s material, then its turnover is too rapid to be detectable using a labelling period of 10 minutes.

To further evaluate the hypothesis that $4\frac{1}{2}$s RNA in infected cells represents an intermediate in the process leading to the formation of 4s RNA, a pulse-chase experiment was carried out. Non-infected and pseudorabies virus infected cells were labelled for 30 minutes with $\left[^{14}C\right]$ uridine or $\left[^{3}H\right]$ uridine respectively, washed and then reincubated for a further 60 minutes in medium 100 μM with respect to non-radioactive uridine.

This procedure, although not leading to immediate rapid dilution of radioactivity in the nucleotide precursor pools, should substantially decrease the specific activity of the radioactively labelled uridine. 'Cytoplasmic' RNA was then extracted from a 1 to 1 mixture of these cells by the cold phenol method.

The result of this type of experiment is shown in Fig. 16. The radioactivity derived from $\left[^{14}C\right]$ uridine - indicative of RNA synthesised in uninfected cells - was found in positions corresponding to ribosomal, 5s and 4s RNA. With the exception of the 5s RNA peak, the labelled material could be correlated exactly with the

Figure 16.

Comparison by Gel Filtration on Sephadex-G100 of Pulse-chased 'Cytoplasmic' RNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells.

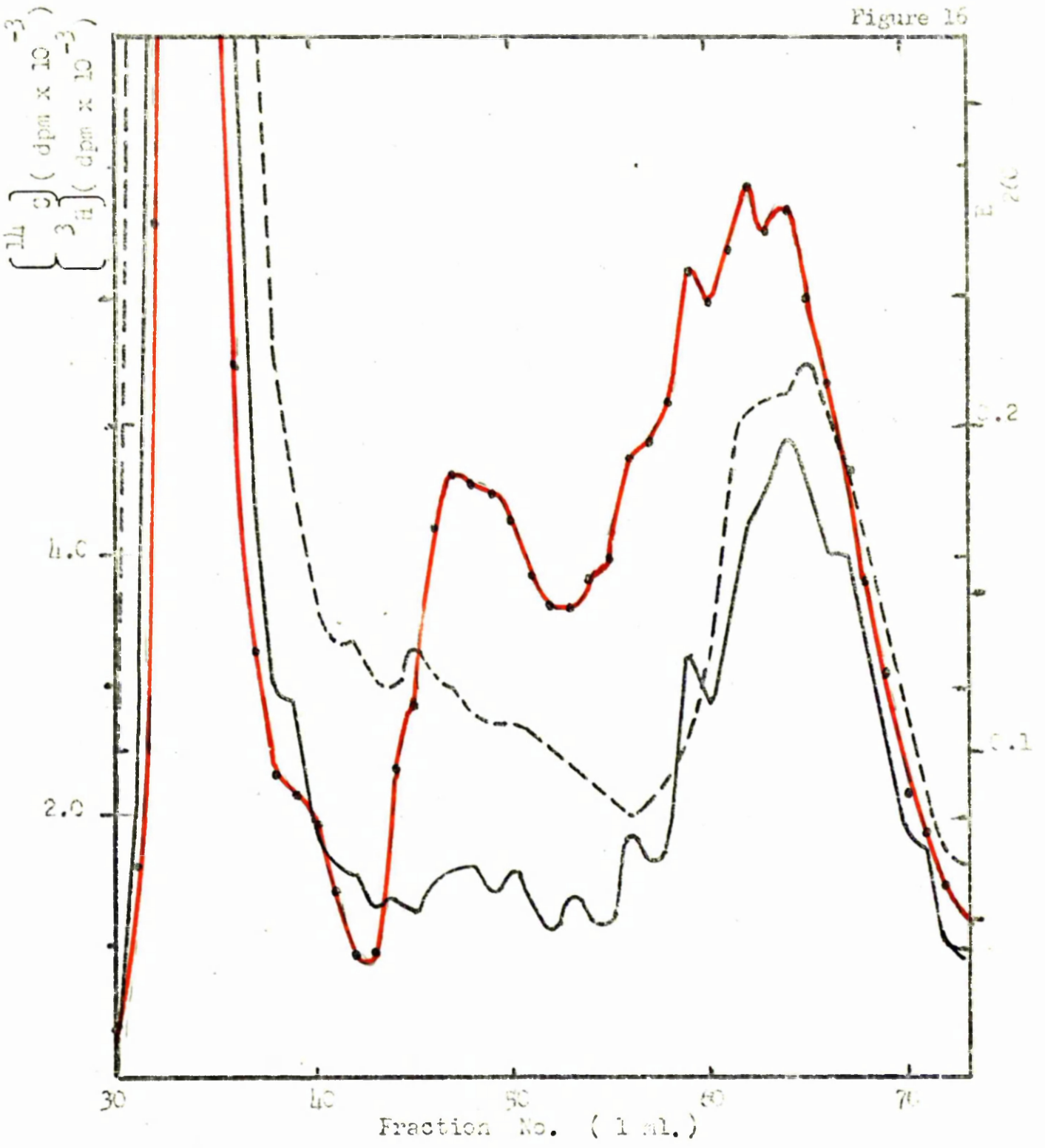
Roller bottle cultures of actively growing BHK21/13 cells were mock infected or infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell and pulse-labelled from 6 - 6 hrs. P. I. with $[^{14}C]$ uridine (G) or $[^{3}H]$ uridine (-5-T) respectively. (500 μC of $[^{3}H]$ uridine of specific activity 8.75 μC/m-mole or 100 μC of $[^{14}C]$ uridine of specific activity 400 μC/m-mole were provided per roller bottle culture). The labelling period was followed by a chase period of 1 hr. before the cells were harvested and pooled. 'Cytoplasmic' RNA was extracted from a 1:1 mixture of these cells and fractionated on a 1.5 x 80 cm. column of Sephadex-G100 at room temperature. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and the total radioactivity associated with each isotope estimated.

– – – E_{260}

——— $[^{3}H]$ disintegrations/min. /fraction.

——— $[^{14}C]$ disintegrations/min. /fraction.

Figure 16


$\left[{}^{14}C\right]$ (dpm x 10^{-3})
$\left[{}^{3}H\right]$ (dpm x 10^{-3})
4.0
2.0
.2
.1
30
40
50
60
70
Fraction No. (1 ml.)

corresponding peaks of U. V. absorbance. This elution profile from pulse-chased uninfected cells completely resembled that obtained on chromatography of similarly prepared pulse-labelled RNA (Fig. 13). This result indicates that none of the species of RNA detected in 30 minutes pulsed uninfected cells are classifiable as transitory intermediates.

The elution profile of $\left[^3H\right]$ label obtained in the co-chromatographic experiment just described - where $\left[^3H\right]$ labelled RNA is derived from the cytoplasm of pulse-chased pseudorabies virus infected cells - also has three main peaks of radioactive material - ribosomal, 5s and 4s RNA. To some extent, the position of all three peaks can be correlated with the corresponding absorbance peaks - in contrast to the situation found on examination of the elution profile of 'cytoplasmic' RNA derived from pulse-labelled pseudorabies virus infected cells. In fact, in RNA from the cytoplasm of pulse-chased infected cells, the distinct $4\frac{1}{2}$s component described in pulse-labelled RNA from infected cells has largely, though not completely, disappeared and has been replaced by a region of radioactive material which has a pronounced leading edge and elutes mainly in the 4s position. A large proportion of the $4\frac{1}{2}$s RNA present in pseudorabies virus

infected cells has therefore been converted by incubation of the cells at 37° for 60 minutes to a species of RNA eluting in the 4s position. If it is accepted that the pronounced leading edge observed on the 4s peak is attributable to continuing synthesis of $[^3H]$ labelled $4\frac{1}{2}$s material, due to the imperfect nature of the chasing conditions, one could infer that in a 60 minutes period all $4\frac{1}{2}$s RNA present is converted to 4s RNA. It is, however, not possible to demonstrate a quantitative conversion of $[^3H]$ radioactivity, which initially eluted in the $4\frac{1}{2}$s region, to material eluting in the region corresponding to 4s RNA after the chase since separate roller bottle cultures are not directly comparable and since some labelled material is undoubtedly synthesised during the chase. Taken over all, the data just presented seem to indicate that one of the steps involved in 4s RNA maturation is inhibited in pseudorabies virus infected cells.

If indeed, such an inhibition does occur, it might be possible to demonstrate differences in modification potential between extracts of non-infected and virus infected cells. Identical preparations of pulse-labelled $[^3H]$ RNA extracted from the cytoplasm of pseudorabies virus infected cells were, therefore, incubated <u>in vitro</u> for 40 minutes at 37° with cell extracts of either

uninfected or pseudorabies virus infected cells under conditions suitable for modification of pre-tRNA. After re-extraction of the RNA and chromatography on Sephadex-G100, the elution patterns illustrated in Fig. 17 (a) and (b) were obtained. Comparison between the distribution of radioactive labels obtained from separate columns must be made by reference to the characteristic optical density patterns.

Examination of the results obtained from infected 'cytoplasmic' RNA pre-treated with infected cell extract (Fig. 17b) indicates that the elution profile of newly synthesised RNA - $[^3H]$label - is completely typical of that obtained for untreated infected cell RNA, both as regards the elution position of the three main peaks and as regards the relative distribution of labelled material among them. More specifically, it should be noted that there still exists RNA eluting from G100 in the $4\frac{1}{2}$s position. On incubation of $4\frac{1}{2}$s RNA with infected cell extract under the conditions used, no alteration in the structure of the RNA leading to a later elution position from G100 has occurred.

The elution profile from G100 of an identical infected RNA preparation, pretreated with uninfected instead of pseudorabies virus infected cell extract, differs from either the uninfected or

Figure 17(a) & (b)

Comparison by Gel Filtration on Sephadex-G100 between 'Cytoplasmic' RNA treated *in vitro* by Enzymes from Non-infected BHK21/13 Cells with Identical RNA treated *in vitro* by Enzymes from Pseudorabies Virus Infected Cells.

Roller bottle cultures of actively growing BHK21/13 cells were infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell and 6 hr. later, pulse-labelled for 30 mins. with [^{3}H]uridine (-5-T) (300 μC of specific activity 4.3 C/m-mole). 'Cytoplasmic' RNA was extracted and either incubated for 40 mins. *in vitro* with a crude extract of non-infected cells (Fig. (a)) or incubated with a similar extract from cells 5 hr. P.I. with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell (Fig. (b)). Incubation conditions are described on p 103. After reisolation, the RNA was fractionated at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0 was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for [^{3}H] emissions associated with trichloracetic acid insoluble material.

– – – E_{260}

——— Non-infected extract ([^{3}H]disintegrations/min./fraction).

——— Virus infected extract

Figure 17 a.

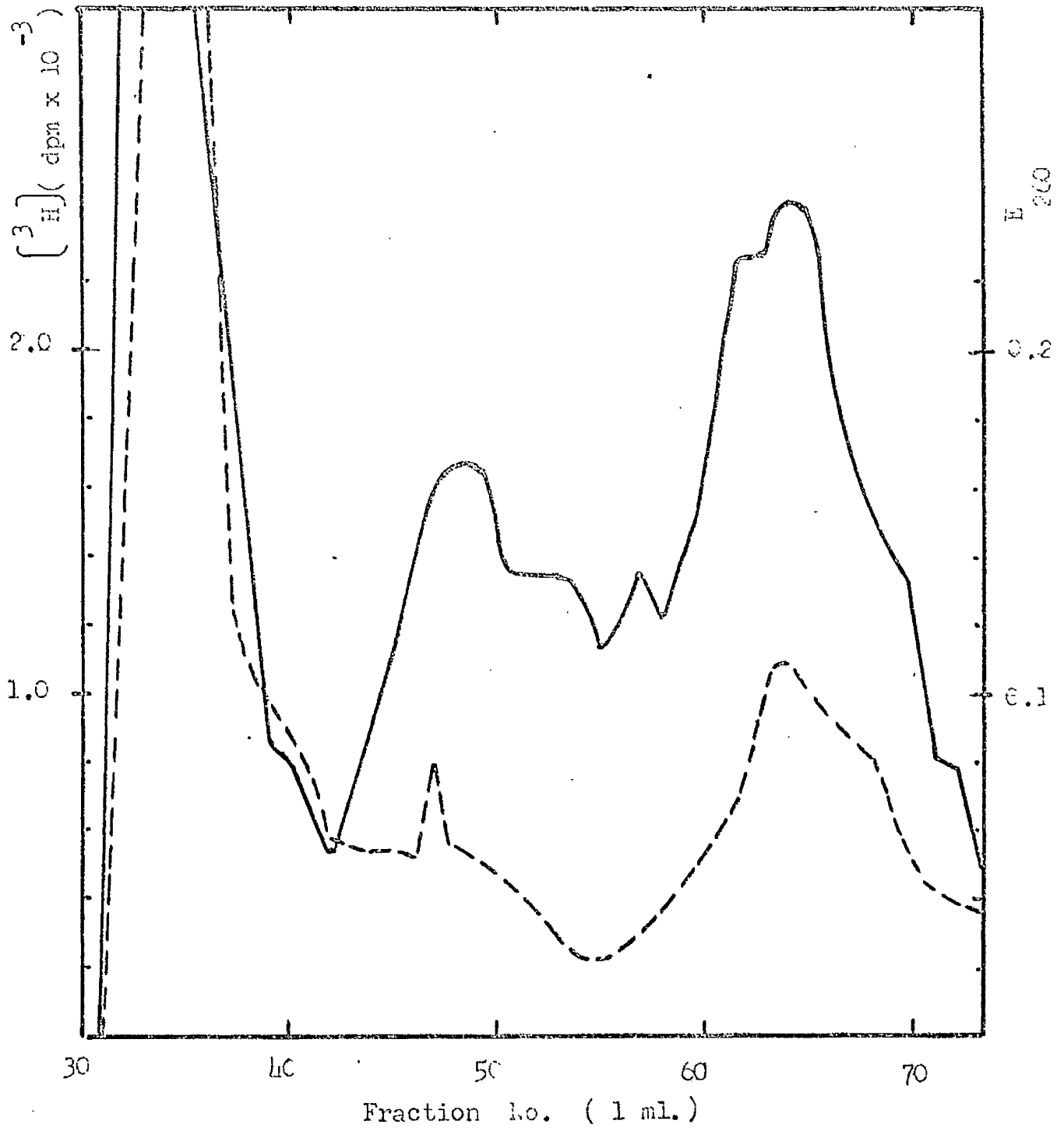
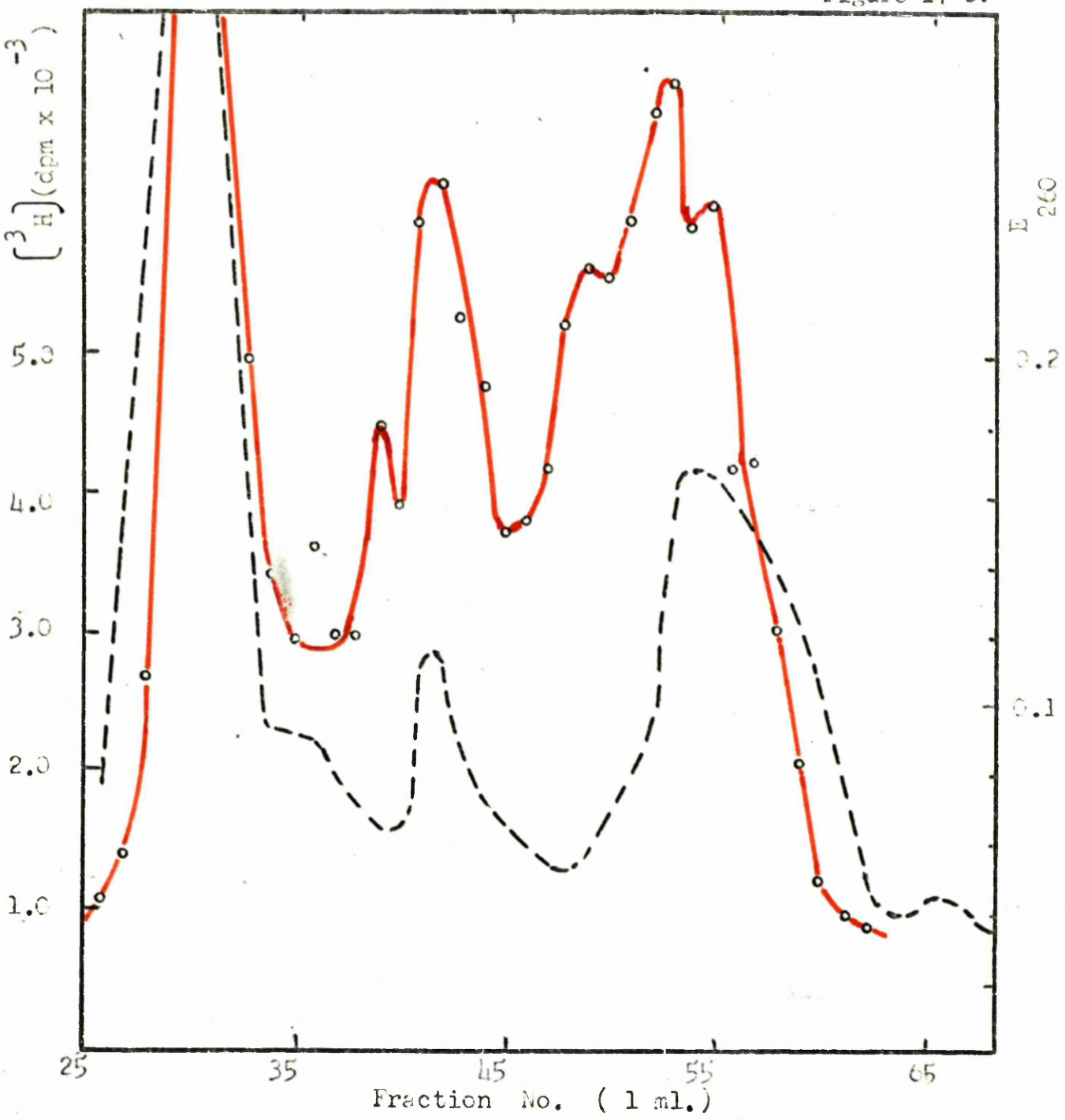


Figure 17 b.


$[^3H]$(dpm x 10^{-3})
5.0
4.0
3.0
2.0
1.0
E_{260}
0.2
0.1
25
35
45
55
65
Fraction No. (1 ml.)

pseudorabies virus infected RNA patterns already described (refer to Fig. 17(a)). Label derived from [^{3}H] uridine is found in three main peaks. In each case, the radioactivity associated with these species could be correlated, as regards elution position, with the corresponding peaks of U. V. absorbance and, therefore, represent ribosomal, 5s and 4s RNA. In this respect, the pattern is similar to those routinely observed for uninfected preparations. The distribution of labelled material among the ribosomal, 5s and 4s RNA peaks, however, resembles that among the ribosomal, 5s and $4\frac{1}{2}$s species present in RNA preparations from pseudorabies virus infected cells. It may, therefore, be concluded that the majority of the $4\frac{1}{2}$s RNA species normally detected in pulse-labelled RNA extracted from pseudorabies virus infected cells is converted, after incubation with uninfected cell extract, to a form which elutes from G100 in the 4s position. But it should be noted, that in both profiles described above, the peaks of U. V. absorbing material are not so sharp as usual and tend to exhibit a slight trailing edge. Maybe some RNase activity had occurred despite the presence of heparin in the incubation mixture. This interpretation should be borne in mind when considering the results presented, although it does not alter any

of the conclusions drawn.

The successful conversion of $4\frac{1}{2}$s RNA to 4s RNA *in vitro* by extracts from non-infected, but not from pseudorabies virus infected cells, raises the possibility that there is a difference in the activity of modifying enzymes in the 2 sources. Since this is equivalent, in essence, to the situation reported in several virus/cell systems for the methylase group of enzymes, investigations were directed along the following lines.

The experiments undertaken involved incubating cells in the presence of non-radioactive uridine and 20 mM-ammonium formate with $[^3H]$ labelled uridine and $[^{14}C]$ methyl methionine. Unlabelled uridine was added merely to lower the specific activity of the labelled material while ammonium formate was necessary, when $[^{14}C]$methyl methionine was used as donor of $[^{14}C]$ methyl groups to RNA, to prevent the equilibration of these methyl groups with the cellular one carbon pool. After G100 column chromatography of the RNA extracted from either uninfected or pseudorabies virus infected cells labelled for 30 minutes, the distribution of both $[^{14}C]$ and $[^3H]$ radioactivity represented in Figs. 18 (a) and (b) was observed. In non-infected cells (Fig. 18 (a)), no incorporation of methyl groups from $[^{14}C]$ methyl methionine is detectable in the

Figure 18(a) & (b).

Comparison by Gel Filtration on Sephadex-G100 of the Distribution of [^{3}H] Uridine and [^{14}C] Methyl Groups in 'Cytoplasmic' RNA from Pulse-labelled (30 mins.) Non-infected and Pseudorabies Virus Infected BHK21/13 Cells.

Roller bottle cultures of actively growing BHK21/13 cells were mock infected (a), or (b) infected with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell and pulse-labelled from 5 - 6 hrs. P.I. simultaneously with [^{3}H] uridine (-5-T) and [^{14}C] methyl methionine. 500 μC of [^{3}H] uridine (specific activity 8.3 C/m-mole) and 100 μC of [^{14}C] methyl methionine (specific activity 25 mC/m-mole) were provided per roller bottle culture. The cells were immediately harvested and RNA extracted from the cell cytoplasmic fraction, was chromatographed at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for the total radioactivity associated with each isotope.

Non-infected RNA

– – – E_{260}

—— [^{3}H] cts./min./fraction

—— [^{14}C] cts./min./fraction

Virus infected RNA

– – – E_{260}

—— [^{3}H] cts./min./fraction.

—— [^{14}C] cts./min./fractio

Figure 18 a.

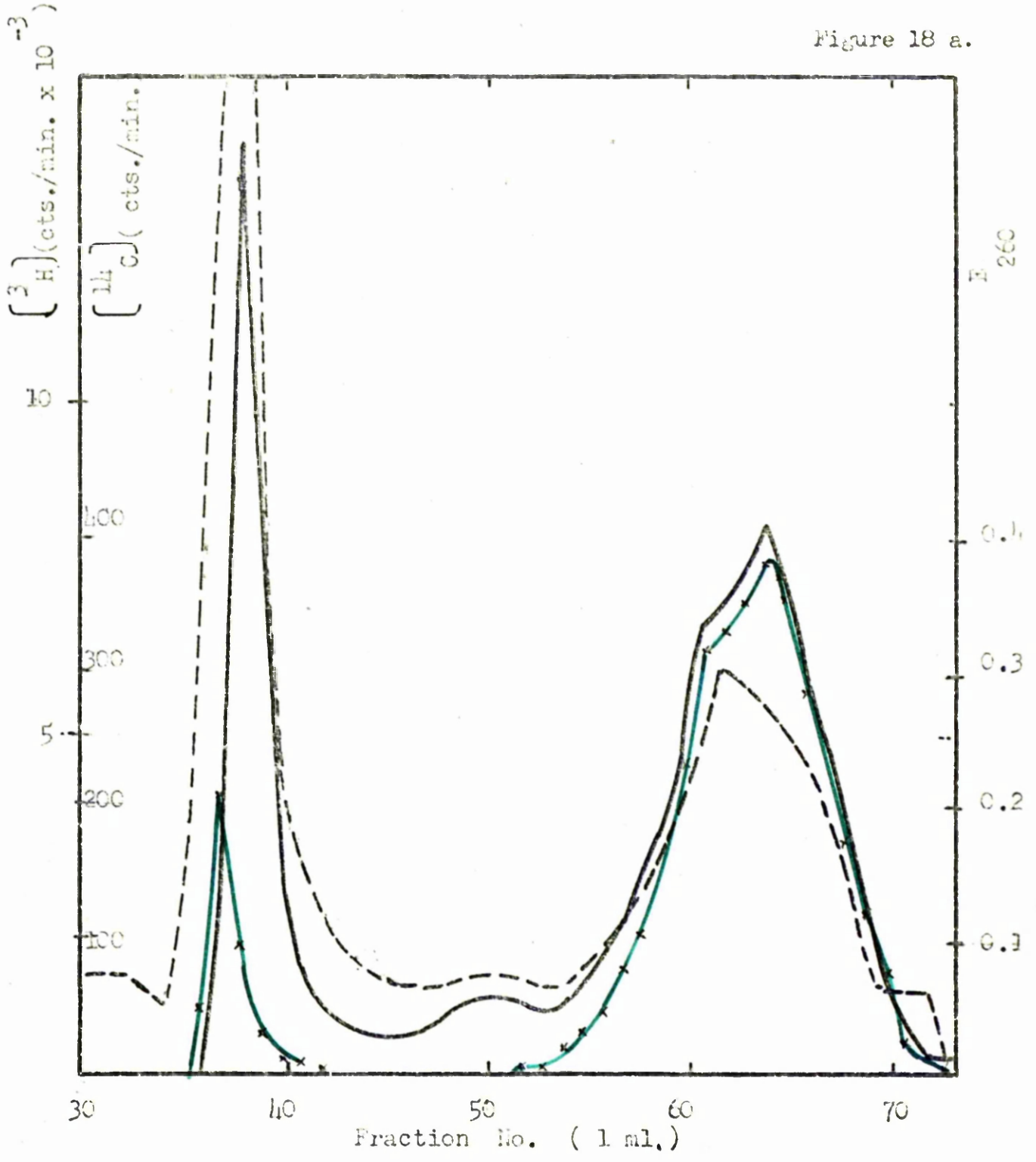
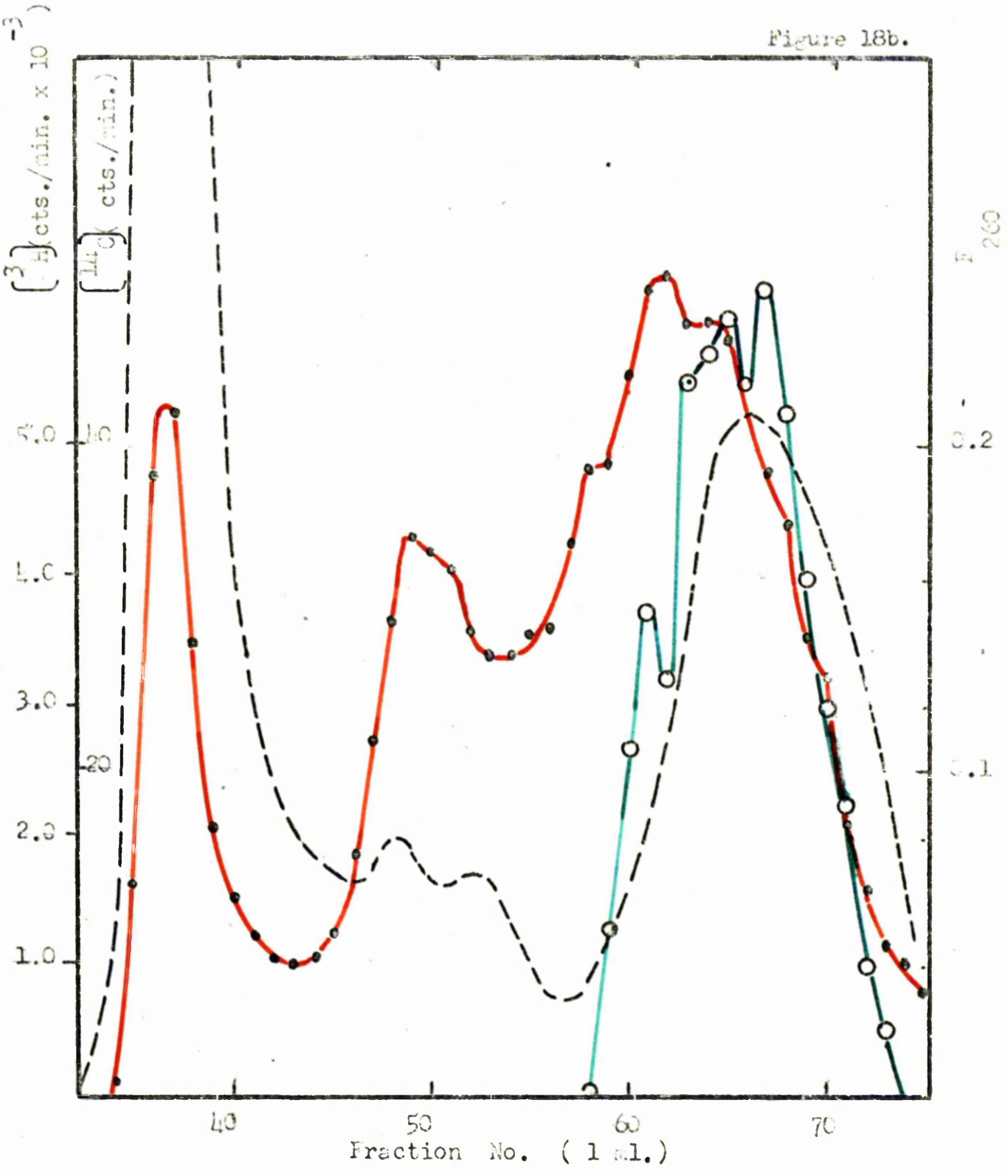

$\left[{}^{3}H\right]$(cts./min. x 10^{-3})
$\left[{}^{14}C\right]$(cts./min.
E_{260}
15
5
400
300
200
100
0.4
0.3
0.2
0.1
30
40
50
60
70
Fraction No. (1 ml.)

Figure 18b.


[3H] cts./min. x 10^-3
[14C] cts./min.)
E 260
3.0
2.0
1.0
0.2
0.1
40
50
60
70
Fraction No. (1 ml.)

5s region but a small proportion is associated with the ribosomal peak and more significantly a relatively high proportion of $[^{14}C]$ methyl groups has been incorporated into 4s RNA material. The distribution of methyl groups corresponds with the profile of absorbance at 260 mμ. Methylated RNA material may, therefore, be identified with the majority of cellular 4s RNA.

The patterns obtained on chromatography of infected preparations confirm the results already obtained in pseudorabies virus infected systems, as regards the relative distribution of $[^{3}H]$ radioactivity and absorbance at 260 mμ and also as regards the depression in 4s RNA synthesis (Fig. 18 (b)). Examination of the $[^{14}C]$ label derived from the $[^{14}C]$ methyl methionine added, reveals 2 major points. Firstly, the radioactivity only elutes in the 4s region, as defined by the absorbance at 260 mμ. $[^{3}H]$ labelled material eluting in the $4\frac{1}{2}$s RNA position is not associated with any $[^{14}C]$ label. Secondly, although it is difficult to draw quantitative conclusions because of the low counts registered, it would appear that methylation of low molecular weight RNA is depressed in the virus infected cells. It could, therefore, be argued that RNA species eluting in the $4\frac{1}{2}$s position represent non-methylated intermediates in the production of 4s RNA and

that since the rate of this conversion at least is depressed in infected cells, $4\frac{1}{2}$s RNA accumulates.

The following observations concerning methylation of ribosomal RNA can also be made.

Reference to Figs. 18 (a) and (b) show that the degree of incorporation of $[^{14}C]$ methyl groups into ribosomal RNA has been reduced by comparison with the controls, it being noticeable that the number of counts collected per fraction was very low. For this reason, it is not possible to determine from this experiment whether the process of infection has really abolished or only diminished the incorporation of $[^{14}C]$ methyl groups into ribosomal RNA. However, when longer incubation periods were used, incorporation of $[^{14}C]$ counts into the high molecular weight RNA fraction was observed in preparations from pseudorabies virus infected cells (Fig. 21). The diminished incorporation of methyl groups may, therefore, be directly correlated with decreased RNA synthesis although, once again, the counts are very low.

Attempts to effect the conversion of the $[^{3}H]$ labelled $4\frac{1}{2}$s RNA component of infected cells to a form eluting at 4s by employing the renaturation method of Lindahl, Adams & Fresco (1966) have been unsuccessful although adoption of a similar technique

by Burdon (1967) was effective in converting putative intermediate forms of 4s i.e. $4\frac{1}{2}$s RNA, to 4s RNA. Separate preparations of $[^3H]$ labelled "infected" RNA were heated for 5 minutes at 60° at pH 7.5 in 20 mM-Tris-HCl, 15 mM-$MgCl_2$ and subjected to gel filtration at either pH 7.0 or pH 5.5. In the former case, little or no radioactive material was found eluting with the void volume - indicating loss of higher molecular weight RNA by precipitation or breakdown - and the labelled material in regions of low molecular weight was unevenly distributed. No conclusions could, therefore, be drawn. On chromatography at pH 5.5 the elution profile for $[^3H]$ labelled infected RNA, pretreated as described, was identical with that obtained when using an identically prepared untreated sample, both being typical of the profiles routinely observed with pseudorabies virus infected preparations. Such a result indicates that renaturation of $4\frac{1}{2}$s RNA does not alter its configuration in a manner leading to an alteration in its elution characteristics. However, the limited data available make this conclusion tentative rather than final.

The detection of a 'new' $4\frac{1}{2}$s species in pseudorabies virus infected cells is not the only deviation observed in this system from the normal pattern of synthesis of low molecular weight RNAs.

As already mentioned, (see Fig. 13) in cells infected for 6.5 hours with pseudorabies virus, there is also an alteration in the distribution of $[^3H]$ uridine label among the three peaks corresponding to ribosomal + m, 4s and 5s RNA. In particular, the ratio of total radioactivity under the 4s peak to that of total radioactivity under the 5s peak is markedly altered falling from a value of 5 or 6/1 for non-infected cellular 'cytoplasmic' RNA to approximately 2/1 in RNA derived from pseudorabies virus infected cell cytoplasm. (Note that with RNA from infected cells the ratio recorded is really $(4 + 4\frac{1}{2})$s RNA/5s RNA). Reference to Table 6 shows that the ratio obtained varies with time post infection.

By 2 - 2.5 hours post infection the value has decreased by a factor of 2 and by 4 - 4.5 hours post infection has reached a value of 1.8/1. No further significant alteration of this ratio occurs, at least up to 8 - 8.5 hours post infection. In order to determine whether the reduction in the value of this ratio is due to an increased synthesis of 5s RNA material or to a decreased synthesis of 4s RNA, the incorporation of $[^3H]$ label into these 3 RNA types at different times after viral infection was examined - see Table 7. All values are expressed as a % of the incorporation

Table 6.

Time (hrs. P.I.)	4s RNA synthesis/5s RNA synthesis
non-infected	5.0
2 - $2\frac{1}{2}$	3.2
4 - $4\frac{1}{2}$	1.84
6 - $6\frac{1}{2}$	1.81
8 - $8\frac{1}{2}$	1.58

Effect of Pseudorabies Virus Infection on the Relative Incorporation in 30 mins. of $[^3H]$ uridine into 4s RNA and 5s RNA.

Non-infected cells or cells infected with pseudorabies virus were labelled with $[^3H]$ uridine at different times P.I. and the cytoplasmic RNA extracted from them separated into 4s, 5s, and high molecular weight RNA by gel filtration on Sephadex-G100 (for details see Fig. 14). The data quoted above for the ratio of $[^3H]$ uridine labelled material present in 4s RNA to that in 5s RNA were obtained from the elution profiles by assessing the areas under the relevant peaks. 4s RNA includes material present in the $4\frac{1}{2}$s position.

Table 7.

Time P. I. (hrs.)	"ribosomal" RNA	"5s" RNA	"4s" RNA
non infected	100%	100%	100%
2 - $2\frac{1}{2}$	23%	184%	73%
4 - $4\frac{1}{2}$	27%	225%	58%
6 - $6\frac{1}{2}$	10%	100%	25%
8 - $8\frac{1}{2}$	16%	170%	32%

Effect of Pseudorabies Virus Infection on the Rates of Synthesis of 4s RNA, 5s RNA and High Molecular Weight RNA.

Non-infected cells or cells infected with pseudorabies virus were labelled with $[^3H]$ uridine at different times P. I. and the cytoplasmic RNA extracted from them separated into its components by gel filtration on Sephadex-G100 (for details see Fig. 14). Radioactively labelled material incorporated in 30 mins. into each of the three species were assessed from the elution profiles by determining the areas under the relevant peaks. After correction for the efficiency of RNA extraction the values were expressed as percentages of the non-infected levels.

into the corresponding type of RNA in non-infected cells after correction for the overall efficiency of RNA extraction had been made, by estimating the E_{260} absorbing material present in the respective ribosomal peaks.

It is obvious that 4s RNA synthesis is diminished in the RNA preparations from pseudorabies virus infected cells and that the degree of inhibition increases with time after infection, levelling off to a value of approximately 30% normal after 8.5 hours post infection. The observed decrease in the $\frac{4sRNA}{5sRNA}$ ratio can, therefore, be attributed, at least in part, to the concomitant diminuation of 4s RNA synthesis.

Examination of the figures estimating the synthesis of 5s RNA material shows that, in contrast to 4s RNA and high molecular weight RNA, the synthesis of the 5s RNA fraction is not inhibited. Indeed, it may be stimulated during the infectious cycle of the virus although the values obtained do not show any clear cut trend - the increase being two-fold and the error in the estimations probably being rather high. However, in view of published reports (Forget & Weissman, 1967 (a)) describing the appearance in KB cells, infected with adeno virus type 2, of a new 5s species of RNA which differs in amount, nucleotide sequence

and cellular location from the host cell 5s RNA, attempts were made to determine the distribution of the newly synthesised component within the cell. Mg^{2+} concentrations were, at all times, sufficient to prevent ribosomal breakdown and a one hour chase period after labelling with $[^3H]$ uridine eliminated complications of accounting for a $4\frac{1}{2}$s component.

The classical distribution picture - as determined in bacterial and mammalian systems - is observed in non-infected cells (See Figs. 19(a) & 19(c)) in that (a) 5s RNA material is only present in the ribosomal fraction (b) 4s RNA material is present in both the ribosomal and supernatant fractions and (c) that the ratio of 4s to 5s material in the ribosomal fraction is approximately 2:1. The distribution of 5s RNA material in pseudorabies virus infected cells is markedly different from that in the non-infected system (Figs. 19(b) & 19(d)), in that, 5s type components are no longer observed only in the ribosomal fraction but are also present in the cell sap fraction. Further, it does not seem likely that the material has been released as a result of ribosomal degradation since there is no evidence for the release of high molecular weight RNA from the ribosomes. The RNA may, therefore, represent a new form of RNA not present in uninfected cells, but further

Figure 19(a), (b), (c) & (d).

Comparison of the Elution Patterns Obtained by Gel Filtration on Sephadex-G100 of Pulse-chased RNA Extracted from Subcellular Fractions of Non-infected and Pseudorabies Virus Infected BHK21/13 Cells.

Roller bottle cultures of actively growing BHK21/13 cells were mock infected or infected at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. 6 hr. later, each culture was provided for 30 mins. with 300 μC of [^{3}H] uridine (-5-T) (specific activity 4.3 C/m-mole). Following a chase period of 1 hr. in unlabelled medium containing 100 mμmoles of uridine per ml., the cultures were harvested and the cells fractionated by differential centrifugation. RNA preparations extracted from cell sap and microsomal fractions were chromatographed at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ and for total [^{3}H] radioactivity. The profiles obtained are shown, the RNA sources being (a) cell sap from non-infected cells, (b) cell sap from virus infected cells, (c) microsomal fraction from non-infected cells and (d) microsomal fraction from virus infected cells.

– – – E_{260}

——— Non-infected material ([^{3}H] disintegrations/min./fraction).

——— Virus infected material

Figure 19 a.& c.

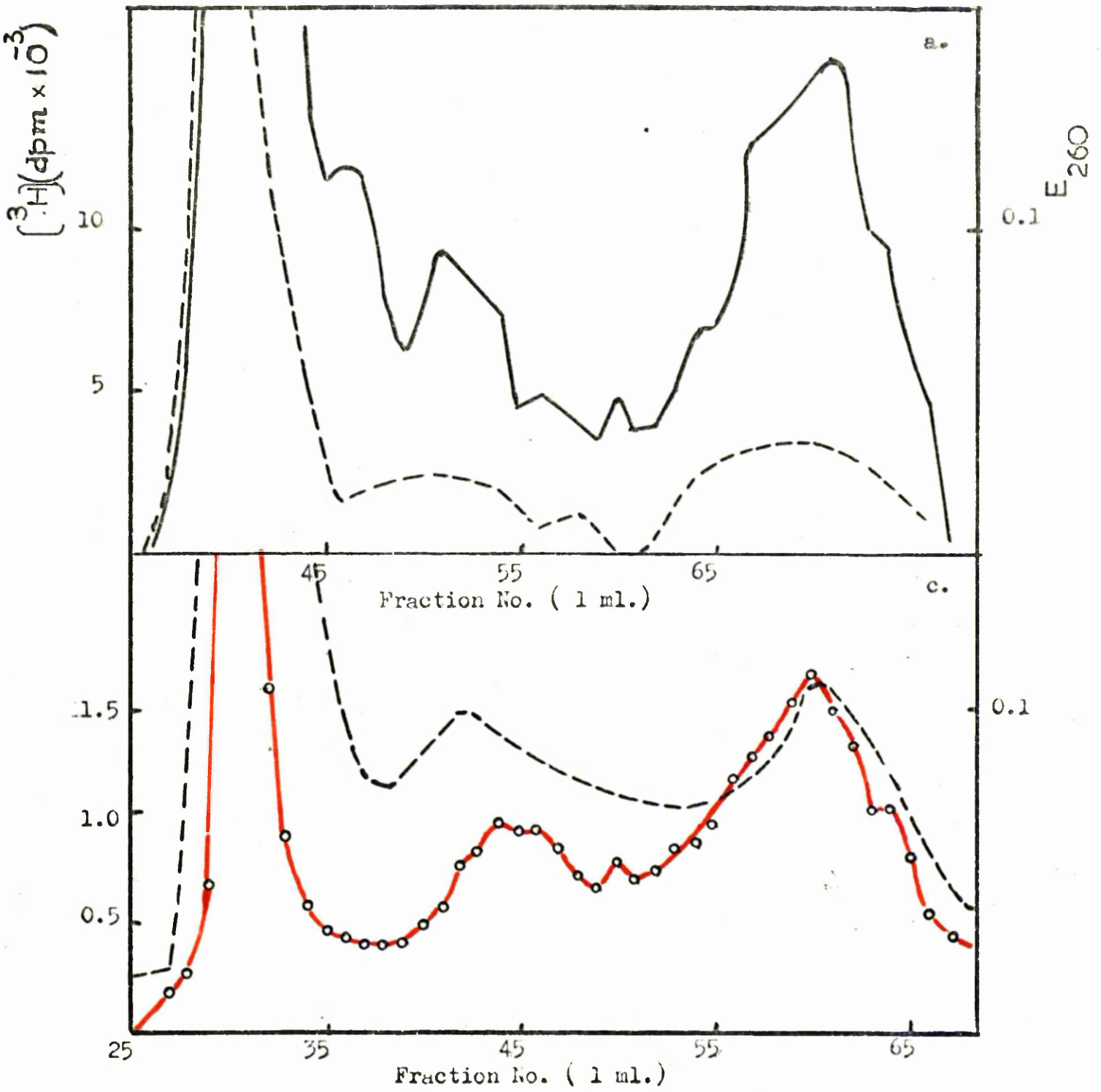
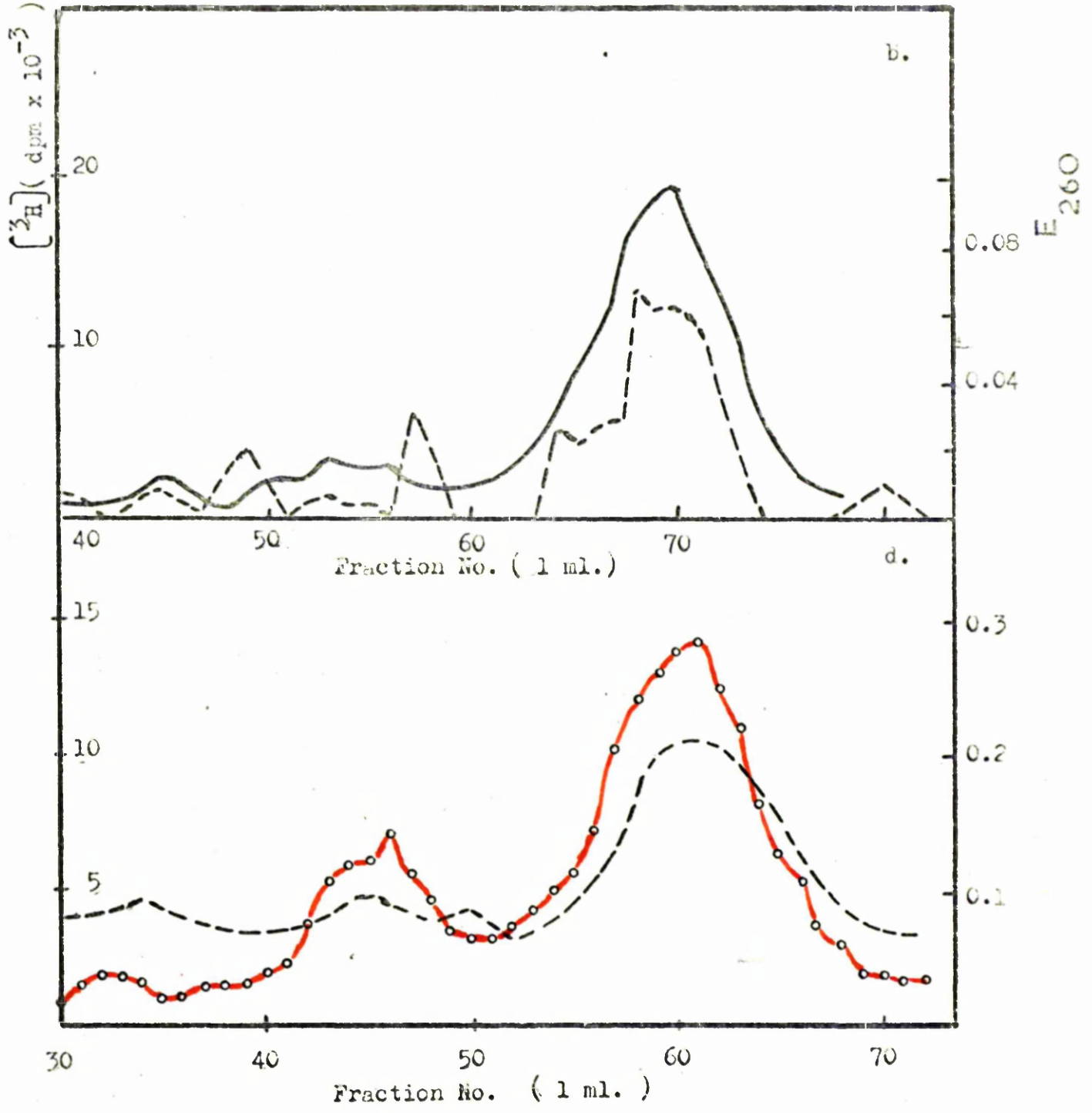

a.
c.
$[^3H](dpm \times 10^{-3})$
E_{260}
10
5
0.1
45
55
65
Fraction No. (1 ml.)
1.5
1.0
0.5
0.1
25
35
45
55
65
Fraction No. (1 ml.)

Figure 19 b.& d.


b.
[3H] (dpm x 10-3)
20
10
E260
0.08
0.04
40
50
60
70
Fraction No. (1 ml.)
d.
15
10
5
0.3
0.2
0.1
30
40
50
60
70
Fraction No. (1 ml.)

interpretation of the data cannot be undertaken in view of the limited knowledge of the mechanism of ribosomal synthesis even in non-infected bacterial systems. No attempt was made to determine whether "infected" 5s RNA material is a structural or a functional component, or whether it is host or virus specified.

4. THE GENETIC ORIGIN OF 4s RNA IN PSEUDORABIES VIRUS INFECTED BHK21/13 CELLS.

Molecular hybridisation is a technique for measuring homology of sequence between 2 polynucleotide chains. In essence, RNA molecules are returned specifically only to those DNA regions, the base sequences of which are identical to the site originally transcribed as RNA. This permits identification of the RNA specifying DNA template. Detection and quantitation of hybrid formation depends upon the conversion of radioactively-labelled RNA of known specific activity into an RNase resistant form. An estimation of the proportion of DNA corresponding to the RNA species in question is obtainable on determination of the amount of RNA bound under conditions of DNA saturation. Such estimates are invalidated either by a failure to reach saturation levels or by the presence of a minor RNA impurity which occupies a much larger proportion of the DNA than the hybrid structure. The

magnitude of the error depends on the percentage of the DNA which specifies the contaminant RNA, compared to that specifying the RNA under investigation. The method is also open to criticism on account of possible heterogeneity of the DNA.

The results obtained by using the DNA-RNA hybridisation technique to identify the genetic origin of 4s RNA synthesised after pseudorabies virus infection are presented below. A short description of the characteristics of the DNA and 4s RNA preparations used is also included since their composition can so profoundly alter the results obtained.

DNAs for use in a DNA-sRNA hybridisation experiment were extracted from BHK21/13 cells and from purified suspensions of pseudorabies virus particles using SDS and a modification of the procedure of Marmur (Marmur, 1961). Pseudorabies virus DNA was, in addition, banded in preparative caesium chloride equilibrium density gradients and respooled in SSC. Discrete bands of viral DNA were obtained on centrifugation and marker cellular DNA, introduced separately into gradient 3, was clearly separated from the viral DNA. In addition, no cellular DNA was observed on the trace obtained by analytical equilibrium gradient centrifugation of the pseudorabies virus DNA preparation in the Spinco Model E

Figure 20.

Analytical Density Gradient Centrifugation of Pseudorabies Virus DNA.

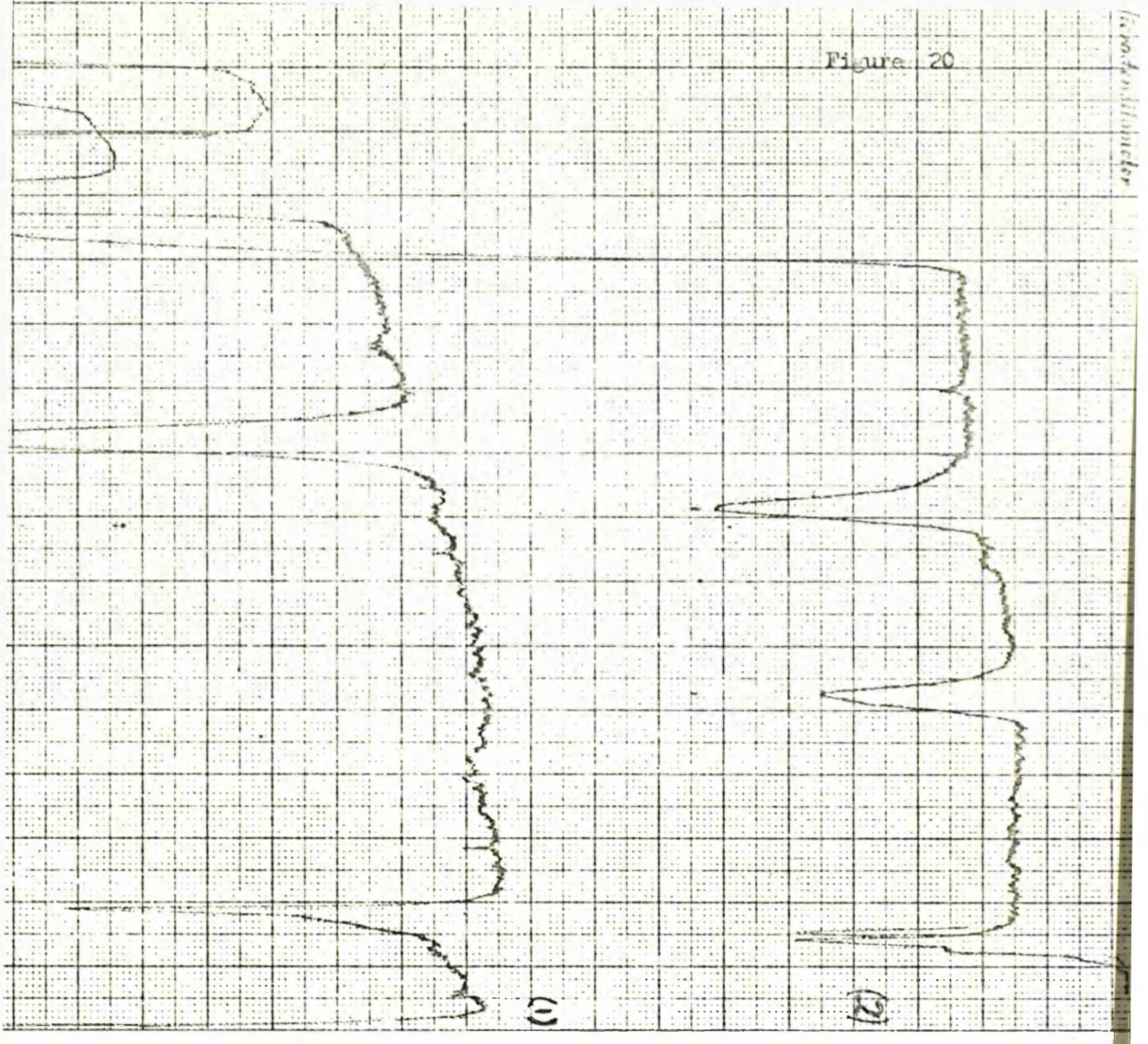
The figure shows a microdensitometer tracing from an analytical density gradient centrifugation in the Spinco Model E Ultracentrifuge of the pseudorabies DNA preparation to be used for molecular hybridisation.

(1) pseudorabies DNA alone

(2) pseudorabies DNA + Cl. Welchii DNA (G+C content 31%) as marker.

28 mm. are equivalent to 40% G+C. The G+C content of the pseudorabies DNA is therefore 71%.

Figure 20



Record No. 696 (1) + (2)

Lever Ratio 1:5

Opt. Magnification:

Slit:

Wedge Range: 0 - 2

Ultracentrifuge (Fig. 20). The pseudorabies virus DNA preparation was, therefore, not contaminated with cellular DNA. Care was taken to ensure that neither DNA preparation exhibited RNase activity.

Radioactively labelled 4s RNA, to be used in molecular hybridisation tests, was derived from the cytoplasmic fraction of uninfected cells and from that of pseudorabies virus infected cells (labelled from 3 - 8 hours post infection) by the method summarised in the legend to Fig. 21. The extraction conditions were designed to reduce the breakdown of high molecular weight RNA, a process which might lead to contamination of the 4s RNA with fragments of mRNA. 4s RNA was separated from high molecular weight RNA and 5s RNA by chromatography on Sephadex-G100 (Fig. 21). In both preparations, ideal fractionation was achieved, each of the bands (ribosomal + mRNA, 5s RNA and 4s RNA) being distinct and well separated from one another. The RNA preparations used were labelled both with $[^{3}H]$ uridine and $[^{14}C]$ methyl groups derived from $[^{14}C]$ methyl methionine. The elution profiles of $[^{3}H]$ labelled material both show absolutely no evidence of RNase activity which, on the basis of random action on RNA, would be expected to lead to fragments of diverse length and, therefore,

Figure 21.

Gel Filtration Profiles on Sephadex-G100 of sRNA Preparations from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells for Use in DNA - sRNA Hybridisation Studies.

Roller bottle cultures of actively growing BHK21/13 cells were mock infected (Fig. (a)) or (Fig. (b)) infected with pseudorabies virus at a multiplicity of infection of 20 PFU per cell and labelled from 3 to 8 hr. P.I. with both $[^3H]$ uridine (-5-T) and $[^{14}C]$ methyl methionine. 500 μC of $[^3H]$ uridine (specific activity 8.75 C/m-mole) and 10 μC of $[^{14}C]$ methyl methionine (specific activity 0.2 mC/m-mole) were provided per roller bottle culture. The cells were immediately harvested and RNA, extracted from the cell cytoplasmic fraction by the cold phenol technique, was chromatographed at room temperature on a 1.5 x 80 cm. column of Sephadex-G100. SSC, pH 7.0, was used as eluant and 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ. The total radioactivity associated with each isotope was determined on 0.05 ml. samples. Fractions between the limits shown were used as source of 4s RNA.

– – – E_{260}

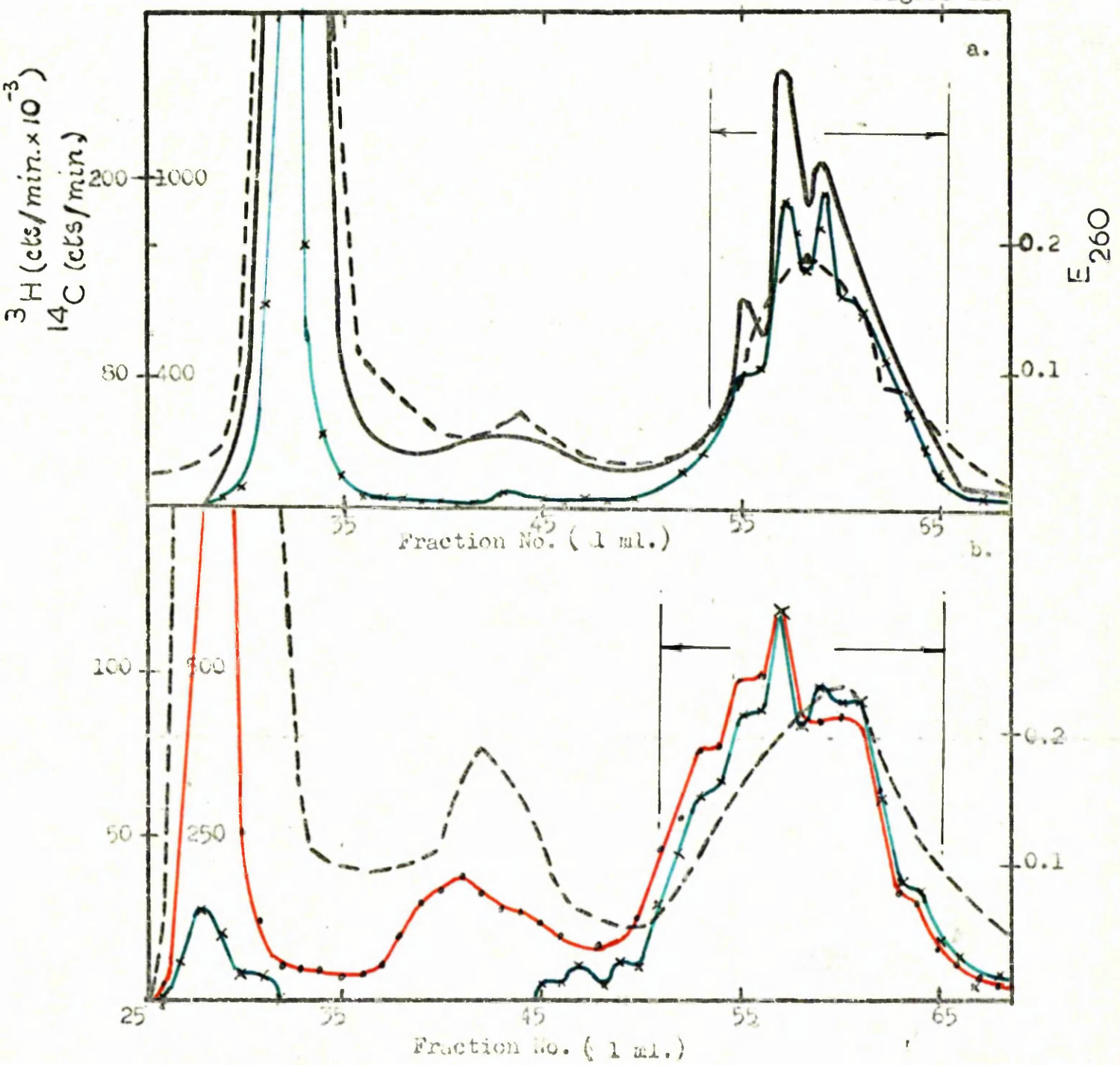
Fig. (a) —— $[^3H]$ cts./min./fraction (non-infected material)

—— $[^{14}C]$ cts./min./fraction

Fig. (b) —— $[^3H]$ cts./min./fraction (virus infected material)

—— $[^{14}C]$ cts./min./fraction.

Figure 21.


a.
^3H (cts/min.×10^{-3})
^{14}C (cts/min.)
200
1000
80
400
0.2
0.1
E_{260}
35
45
55
65
Fraction No. (1 ml.)
b.
100
500
50
250
0.2
0.1
25
35
45
55
65
Fraction No. (1 ml.)

to a wide distribution of radioactivity throughout the elution pattern. Evidence against possible RNase activity is also provided by a degree of correlation between the elution spectra of the 2 isotopes present. In the region of the 4s peak, in both preparations, the ratio of $[^3H]$ uridine to $[^{14}C]$ methyl groups present is constant. This suggests that molecules in this region which are labelled with $[^3H]$ uridine also contain labelled methyl groups and are, therefore, tRNA molecules and not the degradation products of m RNA which would not be methylated. Labelled material present in the 4s regions of these elution patterns was pooled and, after being tested to ensure that no labelled DNA was present, the preparations were used for subsequent DNA-sRNA hybridisation experiments. The preparations had specific activities of 16.35×10^3 counts per minute per μg. (non-infected) and 9×10^3 counts per minute per μg. (virus infected).

The preparations of pseudorabies virus DNA, BHK21/13 DNA, BHK21/13 4s RNA and 4s RNA derived from pseudorabies virus infected cells just described were cross tested for homology by molecular hybridisation by the method of Gillespie & Spiegelman (1965) (p. 109) DNA was efficiently immobilized on membrane filters as demonstrated in a preliminary experiment using

radioactively labelled DNA which determined that 85% of the DNA initially applied to the filters was still attached on the completion of all manipulations. Hybridisation was assayed by incubating filters, loaded with either 20 μg. of pseudorabies virus DNA or 200 μg. of cellular DNA, at various concentrations of the RNA under test and estimating the RNase resistant, $[^3H]$ labelled material associated with them. The results shown in Fig. 22 were obtained. In three of the four cases examined conventional saturation curves were obtained. In the fourth instance, (d), involving hybridisation of control RNA with pseudorabies virus DNA, the values obtained probably represent background due to binding to non-specific DNA as distinct from non-specific binding to the membrane filters. When hybridising "control" RNA to "control" DNA, "infected" RNA to "control" DNA and "infected" RNA to pseudorabies virus DNA the degree of hybridisation increased with increasing concentration of available RNA until saturation values were reached in the presence of approximately 3 μg., 3.5 μg. and 4.5 μg. of the respective RNA per hybridisation mixture. These results, the quantitation of which will be discussed later, demonstrate that virus DNA specifically hybridises with 4s RNA from infected cells but not with RNA from uninfected cells and

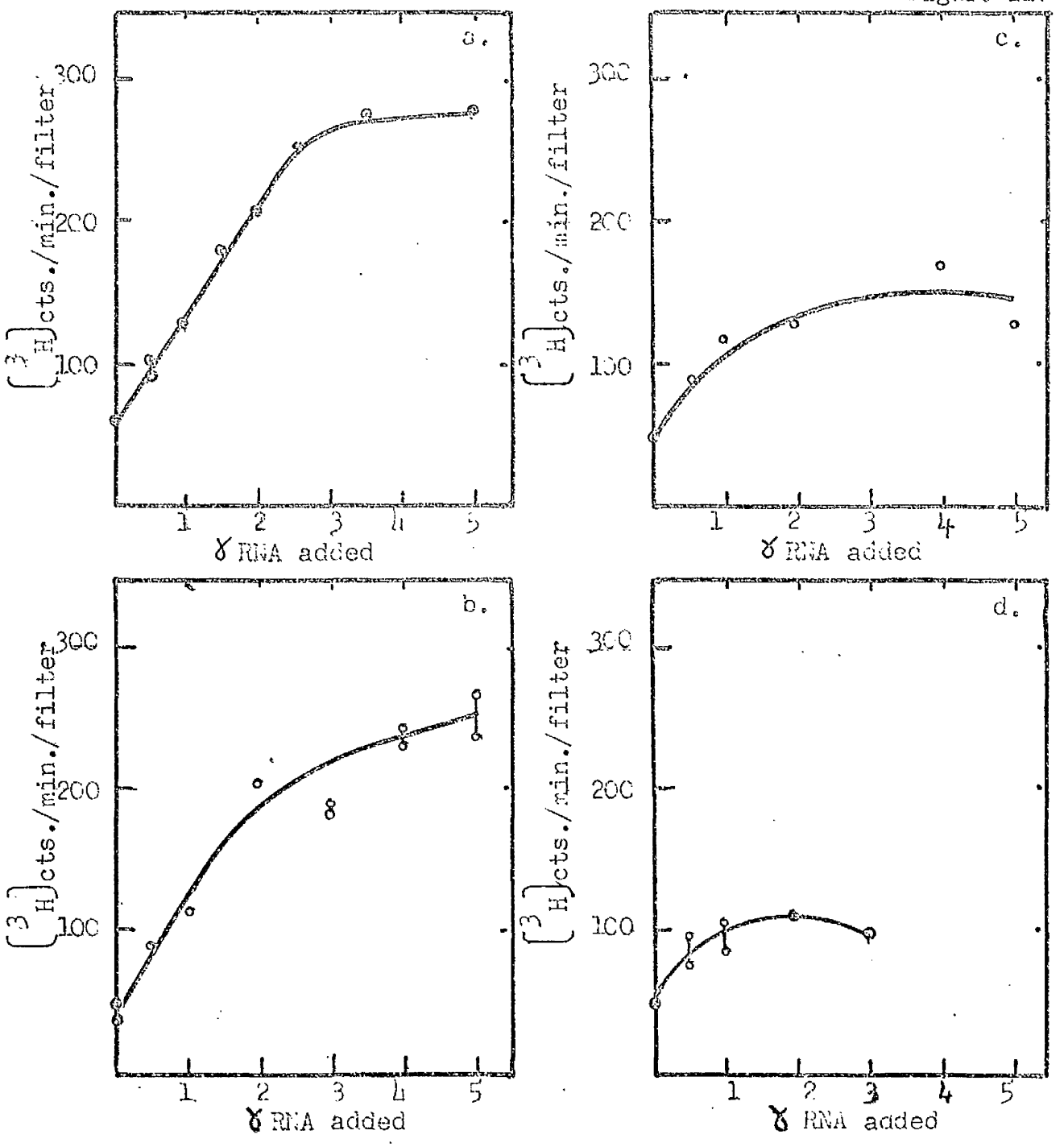
Figure 22.

DNA - sRNA Molecular Hybridisation.

sRNA from BHK21/13 cells and sRNA synthesised in pseudorabies virus infected cells between 3 and 8 hrs. P.I. (as described in Fig. 21) were tested for homology with both pseudorabies and cellular DNA, by the technique of molecular hybridisation. sRNA preparations were labelled with [^{3}H] uridine (-5-T) and hybridisation was detected as RNase resistant, DNA associated [^{3}H] labelled material. The data obtained are shown in the forms of saturation curves in which increasing sRNA concentration in the hybridisation mixtures is plotted against degree of hybridisation, which is estimated as cts./min./filter. Fig. (a) shows the results using sRNA from non-infected cells and 200 μg. of cellular DNA. Fig. (b) shows the results obtained using sRNA from pseudorabies virus infected cell and 20 μg. of pseudorabies virus DNA. (Fig. (c) shows the results obtained using sRNA from pseudorabies virus infected cells and 200 μg. of cellular DNA. Fig. (d) shows the results obtained using sRNA from non-infected cells and 20 μg. of pseudorabies virus DNA.

The RNA preparations were of specific activity 16,350 cts./min./μg. (non-infected) and 9,000 cts./min./μg. (virus infected).

Figure 22.



also that cellular DNA hybridises both with 4s RNA from non-infected and virus infected cells.

If the 4s RNA preparation used can be equated with tRNA (and there is no experimental evidence that it cannot), it can be concluded that certain tRNA species of pseudorabies virus infected cells have been transcribed from the DNA of the invading virus and others from the cellular DNA.

5. THE COMPOSITION OF AMINOACYL tRNA POPULATIONS IN NON-INFECTED AND PSEUDORABIES VIRUS INFECTED BHK21/13 CELLS.

In the light of the preceding two sections and in view of the situation in herpes virus infected cells (Subak-Sharpe, Shepherd & Hay, 1966), it would obviously be interesting to compare the tRNA population in non-infected and pseudorabies virus infected cells. In particular, arginyl, seryl, threonyl and alanyl-tRNAs would be examined. With luck, tRNAs specified by the viral genome or variants of normal species produced as a result of infection (either by undermodification or hyper-modification) might be detected by one of the many chromatographic separation systems available for aminoacyl-tRNAs.

(a) Elucidation of Aminoacylation Conditions.

In order to draw any meaningful conclusions from

column chromatography of aminoacyl-tRNAs or their derivatives, it is necessary to ensure that as far as possible, all species of the tRNA in question become charged with amino acid. The conditions used for attaching the amino acid must, therefore, be such that the aminoacyl-tRNA transferase and the amino acid itself are in excess. These conditions must be determined for "control" enzyme preparations with "control" tRNA and "infected" enzyme preparations with "infected" tRNA.

Fig. 23 shows plots of "aminoacyl-tRNA transferase" concentrations against amino acid acceptance, measured as the incorporation of radioactively-labelled material into trichloracetic acid insoluble material, the amino acids shown being arginine and lysine. Formation of lysyl-tRNA increases with increasing enzymic concentration, for both control and pseudorabies virus infected systems, until 100 μg. of protein are present per reaction mixture while arginyl-tRNA production increases with increasing enzyme concentration until 150 μg. protein has been added. In both cases, further addition of protein does not lead to increased formation of aminoacyl-tRNA. In addition, it

Figure 23.

Arginyl-tRNA Transferase and Lysyl-tRNA Transferase: Effect of Enzyme Concentration.

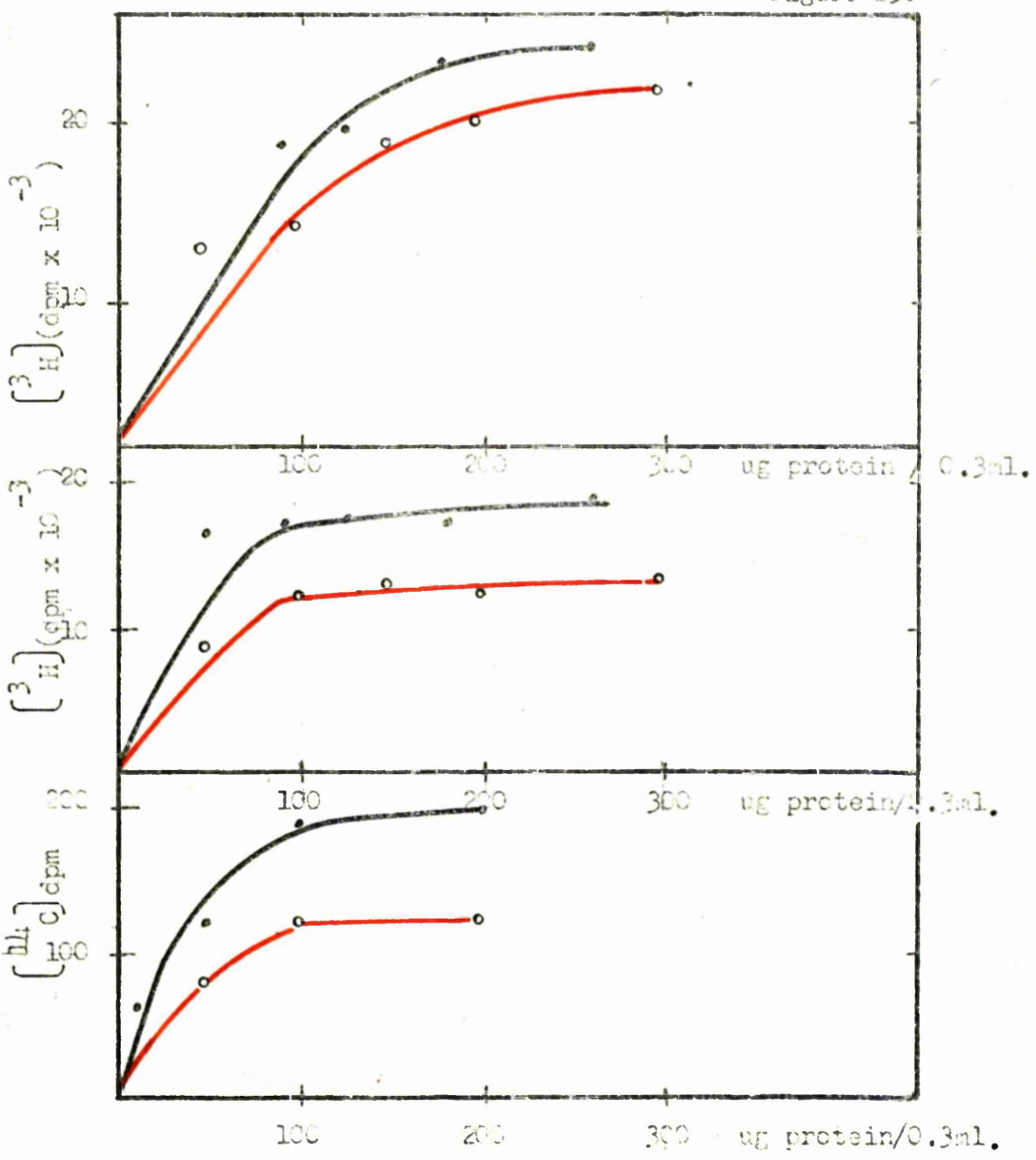
Non-infected BHK21/13 cells and cells 6.5 hrs. P.I. with psuedorabies virus at a multiplicity of exposure of 20 PFU per cell were used as sources of tRNA and aminoacyl-tRNA transferases. 100 µg. of tRNA from each source were loaded with [^{3}H] arginine or [^{3}H] or [^{14}C] labelled lysine using different levels of homologous enzyme preparations. An incubation time of 17 mins. at 37° in a standard reaction mixture (proportionately scaled to 0.3 ml.) was chosen. The reaction was terminated by the addition of trichloracetic acid to 5% after which the radioactivity associated with acid insoluble material was estimated. Enzymic activity, expressed as disintegrations per min. made acid insoluble per assay, was plotted against the concentration of 'enzyme' protein per sample. The labelled material used in Figs. (a), (b) and (c) was respectively [^{3}H] arginine, [^{3}H] lysine and [^{14}C] lysine.

——— Enzyme activity of extract of non-infected cells.

– – – – Enzymic activity of extract of virus infected cells.

(both as disintegrations/min./assay)

Figure 23.


[3H](dpm x 10-3)
[3H](dpm x 10-3)
[14C]dpm
100
200
300
ug protein/0.3ml.
ug protein/0.3ml.
ug protein/0.3ml.

should be noted that the maximum amount of either lysyl- or arginyl-tRNA formed per unit of tRNA is lower (65% normal and 82% normal respectively) if the tRNA used as substrate was derived from pseudorabies virus infected cells. In all subsequent preparative experiments 2 mg. of protein were always used per 1.5 ml. reaction mixture. This value is well in excess of ' at determined in the experiments just described.

Illustrated in Fig. 24 is the plot of arginine concentration against amino acid acceptance, measured as the incorporation of radioactively labelled arginine into trichloracetic acid insoluble material. Formation of arginyl-tRNA increased with increasing arginine concentration for both control and pseudorabies virus infected systems, at least until a concentration of 0.06 mM arginine. It would also appear that by 0.1 mM the addition of further arginine does not lead to further formation of arginyl-tRNA. An amino acid concentration of 0.2 mM was, therefore, used in preparative procedures. As before, it should be noted that the maximum amount of arginyl-tRNA formed per unit of tRNA is lower if the

Figure 24.

Arginyl-tRNA transferase (Group 6.1.1.): Effect of Substrate Concentration.

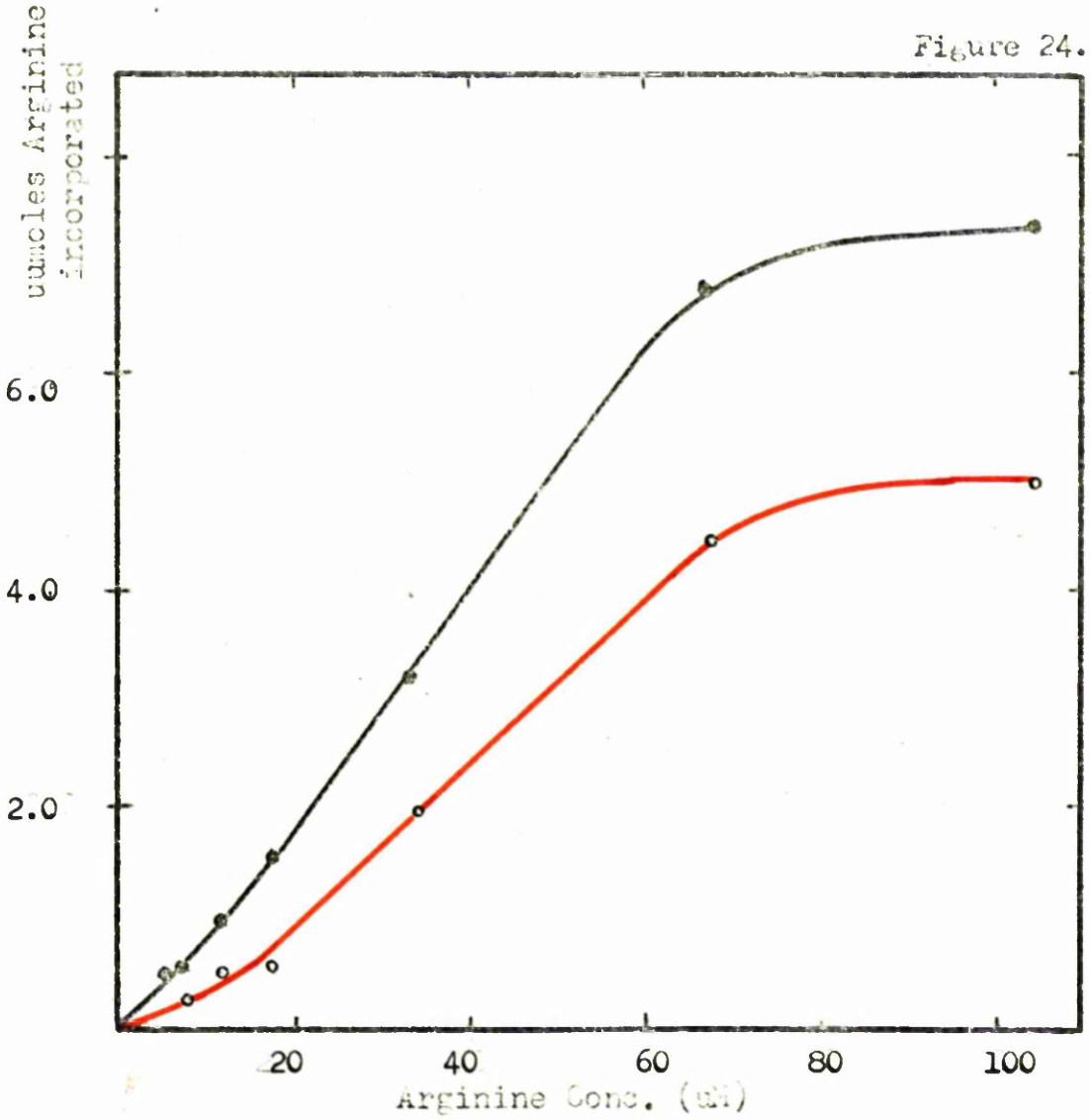
Non-infected BHK21/13 cells and cells 6.5 hrs. P.I. with pseudorabies virus at a multiplicity of exposure of 20 PFU per cell were used as sources of tRNA and aminoacyl-tRNA transferases. 100 μg. samples of tRNA from each source were loaded with [^{3}H] arginine (2 μC/assay) at different overall concentrations of arginine using homologous enzyme preparations. An incubation period of 17 mins. at 37° in a standard reaction mixture (proportionately scaled to 0.3 ml.) was chosen. The reaction was terminated by the addition of ice-cold trichloracetic acid to 5% after which the radioactivity associated with the acid insoluble material was estimated. Enzymic activity, expressed as μμmoles arginine rendered acid insoluble per assay in 17 mins., was plotted against the overall concentration of arginine in the reaction mixtures.

——— Enzymic activity of extracts from non-infected cells.

——— Enzymic activity of extracts from virus infected cells.

(both as uμmoles arginine incorporated per 100 μg. RNA).

Figure 24.


uumoles Arginine
incorporated
6.0
4.0
2.0
20
40
60
80
100
Arginine Conc. (uM)

tRNA used as substrate was derived from pseudorabies virus infected cells. It may also be tentatively concluded that the affinity of the infected enzyme preparation for arginine is lower than that of the control preparation from non-infected cells, although the curves do not show classical reaction kinetics.

Differentially labelled aminoacyl-tRNAs from non-infected and virus infected BHK21/13 cells were examined directly by MAK column chromatography and Reverse phase (type II) chromatography and, as aminoacyl-oligonucleotides, by DEAE cellulose chromatography. The results obtained with each method are discussed separately below.

(b) Comparison of Aminoacyl-tRNA Populations of BHK21/13 Cells and Pseudorabies Virus Infected BHK21/13 Cells by DEAE Cellulose Chromatography of the Products of T_1 RNase Digestion.

If a virus induces the synthesis of new tRNA species, then it would be reasonable to assume that the tRNAs produced differ in their linear sequence of nucleotides from those of the same amino acid recognizing class present in the uninfected cells. The first guanosine residue

in the nucleotide sequence starting from the 3' end might, therefore, occupy a different position in the "viral" species of tRNA from that in the host species. If this is indeed the case, then terminal oligonucleotides produced by extensive RNase T_1 digestion (which hydrolyses exclusively at guanosine phosphate bonds (Sato & Egami, 1957))of tRNA from infected cells will differ in length from those in identical digests of tRNA from uninfected cells. The enzymic attachment of a radioactive amino acid to the end of a tRNA chain provides an effective method of identifying the terminal oligonucleotide fragment from the other oligonucleotides in the digest. Introduction of a double labelling technique (e. g. the infected cell tRNA carrying a $\left[^3H\right]$ labelled amino acid and the control cell tRNA a $\left[^{14}C\right]$ labelled amino acid) also permits the assignment of the terminal oligonucleotides produced as derivatives of either infected cell tRNA or control cell tRNA. Aminoacyl-oligonucleotides can be fractionated according to length by DEAE cellulose chromatography at pH 5. 5 (Herbert, Smith & Wilson, 1964) since at this pH the net charge on any oligonucleotide will be determined mainly by

the number of phosphate residues which it contains. Even if the first guanosine occurs in the viral species of tRNA in the same position as the host tRNA, a relative increase in the peak to which viral tRNA contributes might be observed in cases where aminoacyl-oligonucleotides of more than one length result from RNase T_1 digestion of the radioactively labelled host tRNA. To ensure the validity of results obtained using this technique it is necessary to establish (a) incubation conditions which permit complete RNase T_1 digestion of the aminoacyl-tRNA preparations without excessive loss of the amino acid moeity and (b) chromatography conditions which permit effective separation of oligonucleotides. Herbert, Smith & Wilson (1964) and Ishida & Miura (1965) have reported that at pH 5.5 RNase T_1 is active and retains its specificity for guanosine phosphate bonds. Most aminoacyl bonds are also stable at this pH, the degree of stability depending on the aminoacyl-tRNA in question. Experiments were, therefore, performed in this system to determine the conditions required to achieve maximum hydrolysis of the RNA and the stability, under these conditions, of the

aminoacyl-tRNAs to be used. Chromatography techniques were also checked for efficiency.

(i) Action of T_1 RNase enzyme on tRNA at pH 5.5

Reference to Fig. 25 shows that the hydrolysis of tRNA by RNase T_1 was completed at pH 5.5 at 37° after incubation (for 60 minutes) with 20 units of enzyme per 75 µg. of tRNA (this was determined by measurement of acid-soluble material released) (Takahashi, 1961). Addition of the further 20 units of enzyme after 90 minutes digestion did not result in the release of acid - soluble material thus indicating complete hydrolysis of all RNA present. Release of acid - soluble material did, however, follow the addition of a further 25 µg. of RNA at this time. There must, therefore, still be active enzyme in the digestion mixture. It, therefore, appeared reasonable to use a digestion period of 60 minutes at 37° with 500 units of RNase T_1 per 500 or 1000 µg. of RNA. However, in view of reports of variance in susceptibility of certain bonds to RNase

Figure 25.

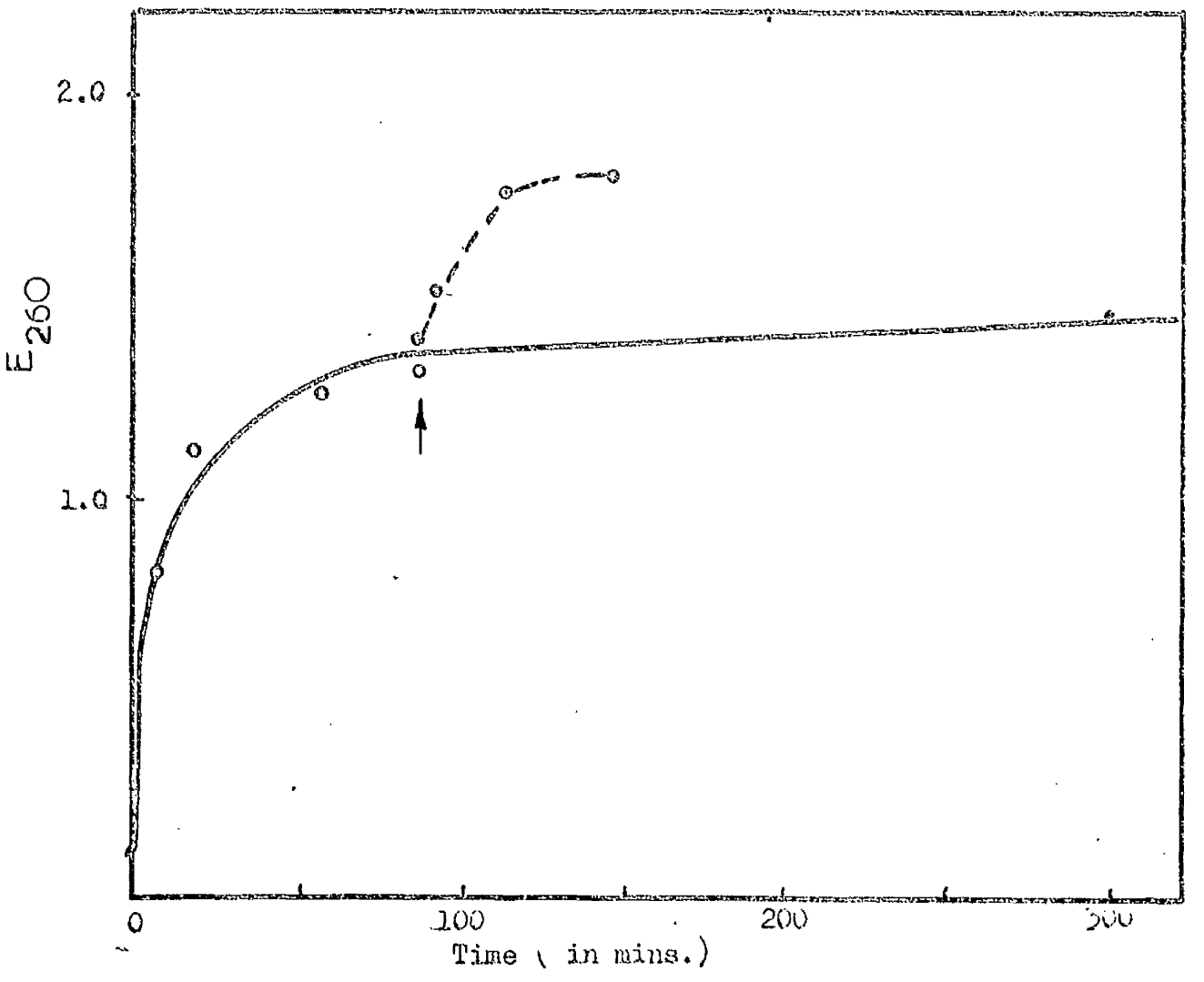
The Release of Acid Soluble Material from tRNA by T_1RNase Digestion.

For each determination, 75 μg. of tRNA was incubated with 20 units of T_1RNase at 37° in 0.5 ml. of sodium acetate buffer, pH 5.5, 2mM-EDTA. At selected times, the reactions were terminated by the addition of ice-cold trichloracetic acid to a final concentration of 5%. Acid insoluble material was removed by centrifugation and the extinction at 260 mμ of the supernatant fluids, diluted to 1 ml., monitored. Enzyme activity, measured as acid soluble material released from tRNA was plotted against incubation time. At the time indicated (↑) a further 25 μg. of tRNA was added to 3 tubes and the incubations continued for the times shown.

——— Acid soluble products from 75 μg. of tRNA.

– – – – Acid soluble products from 75 μg. of tRNA + 25 μg. of tRNA.

Figure 25.



T_1 digestion (Madison, Everett & Kung, 1966b; Herbert & Smith, 1967), before adoption of these conditions for routine experiments it was necessary to ensure that the guanosine phosphate bond nearest to the 3' OH chain terminus was not resistant to hydrolysis under these conditions. Identical preparations of arginyl-tRNA from non-infected BHK21/13 cells were incubated with RNase T_1 using the procedure outlined in the methods section for 5 minutes, 30 minutes, 60 minutes, 2 hours and 4 hours prior to chromatography on DEAE cellulose. All the profiles obtained are shown in Fig. 26. Analysis of these chromatographic patterns, neglecting that for 2 hour digestion, shows that the number and relative proportion of arginyl-oligonucleotide species do not vary substantially with RNase T_1 digestion time and that only after a 5 minute digestion period is there any evidence of undigested tRNA molecules (as counts eluting at 1 M-ammonium formate). The % of the radioactive material initially applied to the column which eluted as arginyl-oligonucleotides was,

Figure 26.

The Release of 5' Terminal Oligonucleotides from [^{14}C] Arginyl-tRNA by T_1RNase Digestion.

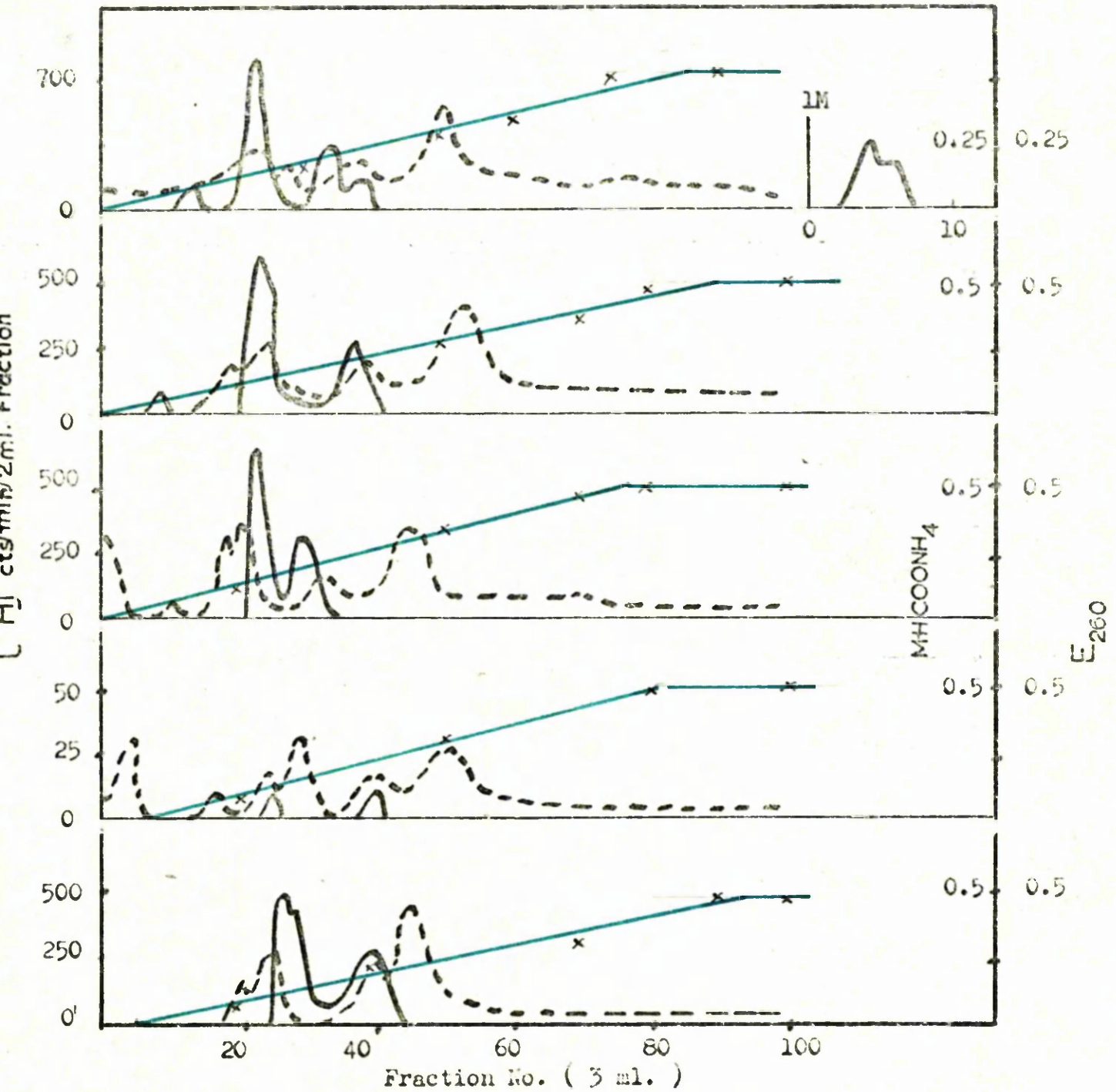
500 µg. of [^{14}C] arginyl-tRNA containing 2 x 10^3 cts. /min. /mg. was incubated with 500 units of T_1RNase at 37° in sodium acetate buffer, pH 5.5, 2mM with respect to EDTA for a) 5 mins. b) 30 mins. c) 1 hr. d) 2 hrs. and e) 4 hrs. The reactions were terminated by the addition of 90% phenol, 0.1% 8 hydroxy-quinoline. After deproteinisation, the arginyl-oligonucleotides were chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate, pH 5.5, (from 0.01M-0.4M) was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ, salt concentration and total [^{14}C] radioactivity. Subsequent batch elution with 1M ammonium formate, pH 5.5, was also carried out.

– – – E_{260}

——— [^{14}C] cts. /min. /2 ml. of fraction.

——— ammonium formate concentration.

Figure 26.


700
0
1M
0.25
0.25
0
10
500
250
0
0.5
0.5
500
250
0
0.5
0.5
MH COONH4
50
25
0
0.5
0.5
500
250
0
0.5
0.5
20
40
60
80
100
Fraction No. (3 ml.)
E260

however, decreased from 85% after 1 hour incubation to 65% after 4 hours incubation. It was, therefore, deemed advisable to use a one hour incubation period, at which time the balance between extent of T_1 RNase digestion and loss of amino acid counts was most favourable.

(ii) Stability of Aminoacyl-tRNA

The results shown in Table 8 indicate that arginyl, seryl, prolyl and alanyl-tRNAs derived from either uninfected or pseudorabies virus infected BHK21/13 cells are relatively stable to incubation for 1 hour, at 37° in 100 mM - sodium acetate buffer pH 5.5, 2 mM with respect to EDTA. The stability of the different aminoacyl-tRNAs seemed relatively constant for this period, approximately 5% of the counts being lost on average.

(iii) Resolution by DEAE Cellulose Chromatography

The U.V. extinction profile obtained after DEAE cellulose chromatography of a mixture of ATP and AMP with a linear elution gradient of ammonium formate pH 5.5, consists of 2 peaks - as shown in Fig. 27.

Comparison of the Stability of Certain Aminoacyl-tRNA Species.

Aminoacyl-tRNA species, radioactively labelled *in vitro* in the aminoacyl moieties were either incubated for 1 hr. at 37° in 100 mM-sodium acetate buffer 2 mM - EDTA (treated) or maintained at 4° for 1 hr. in identical buffer (untreated). Estimates of the labelled amino acid attached to tRNA were mad by the standard trichloracetic acid precipitation method. The stability of several aminoacyl-tRNAs from both non-infected and pseudorabies virus infected, 6.5 hrs., BHK21/13 cells were examined.

Table 8.

Aminoacyl tRNA	RNA Source	cts./min./sample (Untreated)	(Treated)
Alanyl-tRNA	Non-infected BHK21/13 cells	1704	1415
Alanyl-tRNA	Viral infected BHK21/13 cells	2525	2230
Seryl-tRNA	Non-infected BHK21/13 cells	4500	4010
Seryl-tRNA	Viral Infected BHK21/13 cells	4890	4800
Lysyl-tRNA	Non-infected BHK21/13 cells	320	250
Lysyl-tRNA	Viral Infected BHK21/13 cells	990	900
Arginyl-tRNA	Non-infected BHK21/13 cells	330	355
Arginyl-tRNA	Viral Infected BHK21/13 cells	3000	2500

Figure 27.

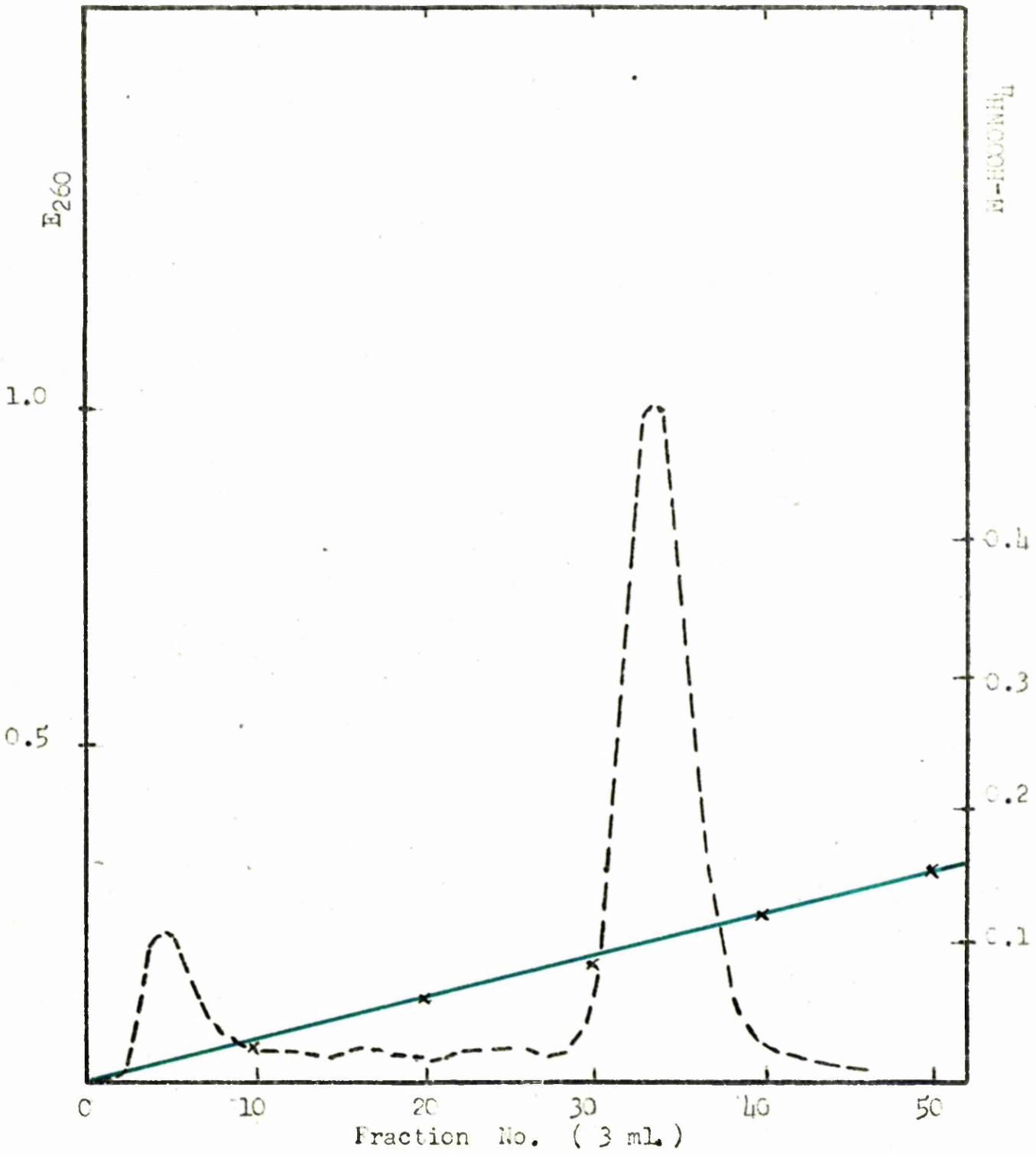
Elution Pattern Obtained by DEAE Cellulose Chromatography of a Mixture of ATP and AMP.

500 μg. of ATP mixed with 400 μg. of AMP were chromatographed on a 1 x 5 cm. column of DEAE cellulose at 4° using a linear ammonium formate gradient at pH 5.5. Determinations of the extinction at 260 mμ and of the ammonium formate concentration were made on the 3 ml. fractions collected.

– – – E_{260}

——— Ammonium formate concentration (expressed as molarity)

Figure 27.



These peaks are well separated and elute at 15 mM and 100 mM-ammonium formate, the former being attributable to AMP and the latter to ATP. It, therefore, appears that molecules bearing a net negative charge of 3 units elute at 100 mM-ammonium formate.

(iv) Dual Labelled Experiments

T_1 RNase digestion of doubly labelled tRNA preparations of arginyl-, seryl-, lysyl- and alanyl-tRNA followed by DEAE cellulose chromatography of the products have been carried out under the conditions already outlined. It should be noted that recent evidence obtained by Bell (Bell, 1969) suggests that the elution of aminoacyl-oligonucleotides from DEAE cellulose is significantly delayed at pH 5.5 (cf. pH 7.0). At present, it is difficult to reconcile these observations with the data to be reported.

Arginyl-tRNA

Fig. 28 shows an elution pattern obtained after DEAE cellulose chromatography of the components of a T_1 RNase digest of a mixed RNA preparation from

Figure 28.

Comparison of Arginyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography of T_1RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. after infection at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with [^{3}H] arginine (non-infected) and [^{14}C] arginine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1RNase digestion products of 260 µg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate buffer, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ, for salt concentration and for total radioactivity associated with each isotope.

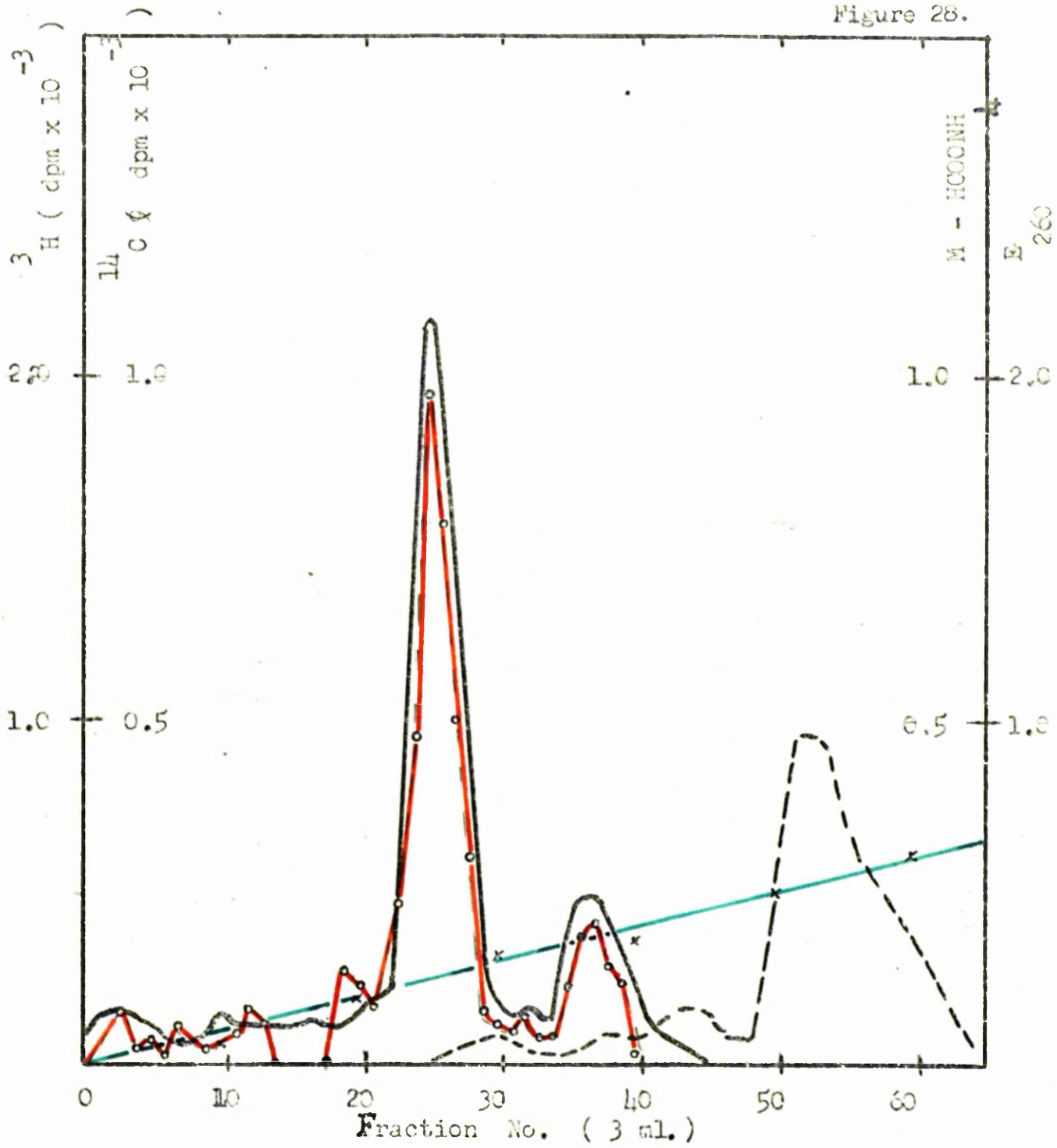
– – – E_{260}

——— [^{3}H] disintegrations/min. / 2 ml. of fraction.

——— [^{14}C] disintegrations/min. / 2 ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 28.


3H (dpm x 10^-3)
14C (dpm x 10^-3)
M - HCOONH4
E 260
2.0
1.0
1.0
0.5
1.0
0.5
1.0
0
10
20
30
40
50
60
Fraction No. (3 ml.)

uninfected and pseudorabies virus infected BHK21/13 cells which had previously been maintained in high serum medium. RNA had been previously labelled with [^{3}H] arginine(non-infected)and [^{14}C] arginine (infected). The fractions were eluted with a linear (0.01M-0.5M) gradient ammonium formate pH 5.5. In addition to U.V. absorbing peaks, there are two distinct peaks of radioactivity. These contain both [^{14}C] and [^{3}H] tracer and elute at 120 mM (Comp. I) and 180 mM (Comp. II) ammonium formate. There is no evidence of any [^{14}C] labelled material eluting by itself and so there would not appear to be present in the infected preparation any arginyl-tRNA species the terminal base sequence of which differs from that of those present in the control cells. The ratio of total radioactivity (Comp. I/II) in the peaks centred at 120 mM and 180 mM-ammonium formate is 3.5/1 in the infected preparation and 3.0/1 in the control. These values seem to be identical within the limits of experimental error. Therefore, under the conditions used,there is no difference detectable by this method between the populations of arginyl-tRNA

in uninfected and 6 hours pseudorabies virus infected cells. The chromatographic pattern obtained when the radioactive labels were reversed showed no basic difference from that illustrated in Fig. 28.

Illustrated in Fig. 29 is the chromatographic profile, obtained by an identical procedure to that outlined above, using as starting material sRNA isolated from cells (uninfected or pseudorabies virus infected) in which metabolism had been depressed by deprivation of serum. Once again, the non-infected $[^{3}H]$ pattern and the virus infected $[^{14}C]$ pattern are superimposable and have two distinct peaks eluting at 125 mM and 175 mM-ammonium formate. The ratio of total radioactivity (Comp. I/II in the peaks centred at these molarities is unchanged by pseudorabies virus infection, being 3.4/1 for the non-infected preparation and 3.34/1 for the infected . Similar values were obtained in the previous experiment and so it would seem that the populations of arginyl-tRNA in dividing BHK21/13 cells, metabolically inactive cells

Figure 29.

Comparison of Arginyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography of T_1 RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from low serum non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^3H]$ arginine (non-infected) and $[^{14}C]$ arginine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1 RNase digestion products of 750 µg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate buffer, pH 5.5, was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ, for salt concentration and for total radioactivity associated with each isotope.

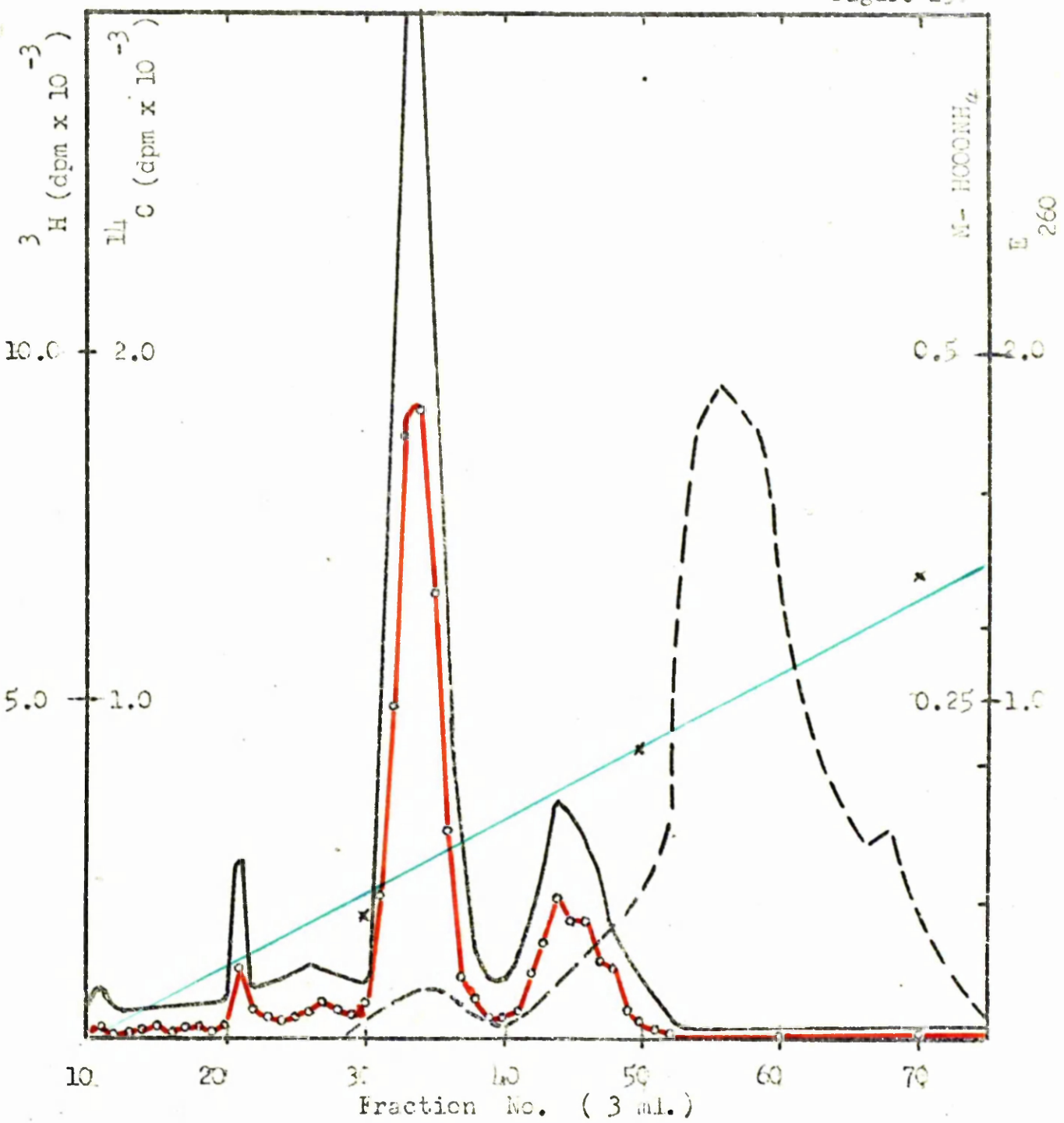
– – – E_{260}

——— $[^3H]$ disintegrations/min. /2 ml. of fraction.

——— $[^{14}C]$ disintegrations/min. /2ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 29.


3H (dpm x 10^-3)
14C (dpm x 10^-3)
M- HCOONH4
E 260
10.0
2.0
5.0
1.0
0.5
0.25
10
20
30
40
50
60
70
Fraction No. (3 ml.)

and pseudorabies virus infected cells are identical. A similar experiment with reversed labels again gave identical superimposable patterns for non-infected and infected preparations. The ratio of Comp. I/II in this case was 2.5/1 for both populations.

Lysyl-tRNA

The pattern obtained by linear gradient elution (with ammonium formate) from DEAE cellulose of the components of a T_1 RNase digest of a mixedly labelled RNA preparation from uninfected, $[^3H]$ label, and pseudorabies virus infected, $[^{14}C]$ label, BHK21/13 cells is shown in Fig. 30. 2 peaks of radioactivity, 1 major and 1 minor, containing both $[^{14}C]$ and $[^3H]$ counts elute at 80 mM and 160 mM-ammonium formate. There is no suggestion of any peak containing only a single isotope. The ratio of the total radioactivity in the peak centred at 80 mM to that in the peak centred at 160 mM is 3.2/1 in the case of the control and 2.9/1 in the case of the infected. That is to say, the values are identical within the limits of experimental error. These results indicate that neither are the proportions

Figure 30.

Comparison of Lysyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography of T_1 RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^3H]$ lysine (non-infected) and $[^{14}C]$ lysine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1 RNase digestion products of 1 mg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate buffer, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mμ, for salt concentration and for total radioactivity associated with each isotope.

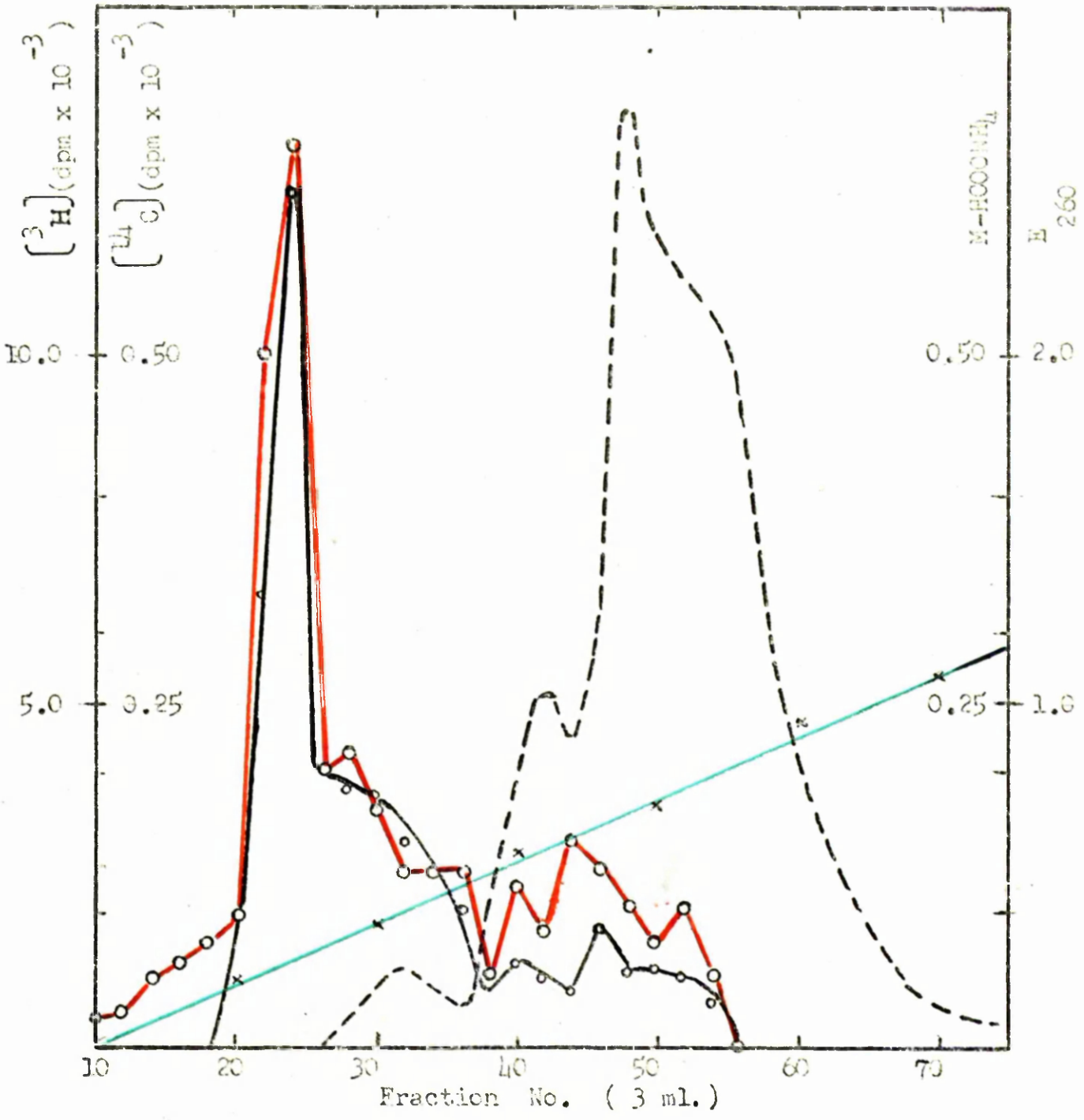
– – – E_{260}

——— $[^3H]$ disintegrations/min. /2 ml. of fraction.

——— $[^{14}C]$ disintegrations/min. /2 ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 30.


$[^3H]$ (dpm x 10^{-3})
$[^{14}C]$ (dpm x 10^{-3})
M-HCOONH$_4$
E_{260}
10.0
0.50
5.0
0.25
0.50
2.0
0.25
1.0
10
20
30
40
50
60
70
Fraction No. (3 ml.)

of the lysyl-tRNA species in BHK21/13 cells altered nor are any new species produced after pseudorabies infection.

Seryl-tRNA

The profile obtained by DEAE cellulose chromatography of the products of a T_1 RNase digest of a mixedly labelled RNA preparation from uninfected, $[^{14}C]$, and pseudorabies virus infected, $[^{3}H]$, BHK21/13 cells, illustrated in Fig. 31 shows a single peak of $[^{3}H]$ and $[^{14}C]$ radioactivity eluting at 60 mM-ammonium formate. The $[^{14}C]$ and $[^{3}H]$ patterns are not exactly superimposable since there is a slight trailing edge on the control preparation. This probably is not significant. More important, however, is the fact that here also there is no evidence for any peak of radioactivity containing one isotope only. The significance of the single point peak of both $[^{3}H]$ and $[^{14}C]$ radioactivity in fraction 2 is not understood, although similar single point fractions are occasionally observed. Identical patterns are obtained when the control tRNA is labelled with $[^{3}H]$ serine and the virus infected tRNA carries

Figure 31.

Comparison of Seryl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography of T_1RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^{14}C]$ serine (non-infected) and $[^{3}H]$ serine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1RNase digestion products of a total of 1 mg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate buffer, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mμ, for salt concentration and for total radioactivity associated with each isotope.

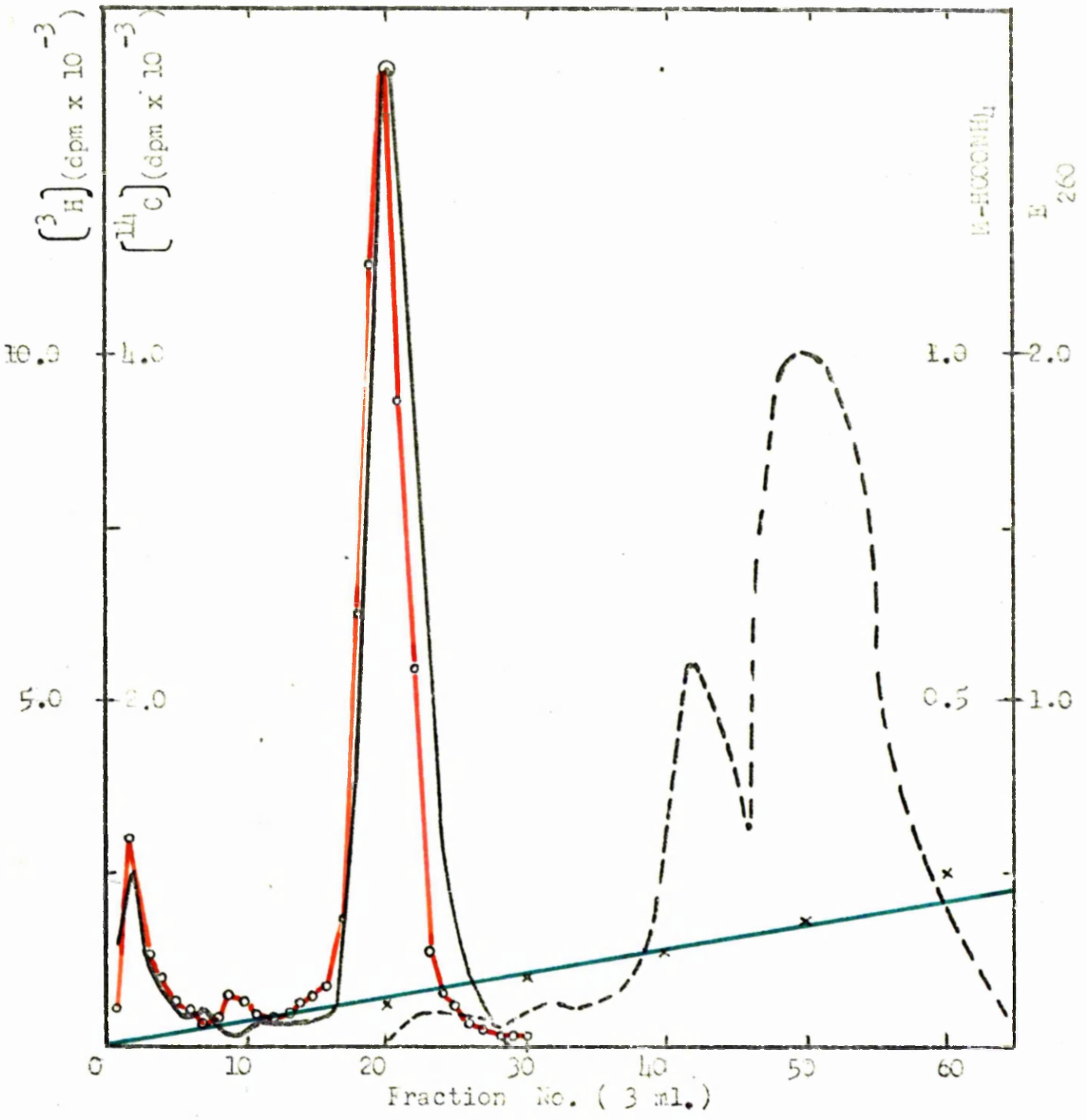
--- E_{260}

——— $[^{3}H]$ disintegrations/min. /2 ml. of fraction

. . . . $[^{14}C]$ disintegrations/min. /2 ml. of fraction

——— Ammonium formate concentration (expressed as molarity).

Figure 31.


$[^3H]$ (dpm x 10^{-3})
$[^{14}C]$ (dpm x 10^{-3})
H-HCOONH$_4$
E_{260}
10.0
4.0
5.0
2.0
1.0
2.0
0.5
1.0
0
10
20
30
40
50
60
Fraction No. (3 ml.)

$[^{14}C]$ labelled serine.

Therefore, under the conditions used, no species of seryl-tRNA, the terminal base sequence of which differs from that normally present in the host cells, is detected.

Alanyl-tRNA

Examination of the chromatographic patterns of oligonucleotides produced by T_1 RNase digestion of mixedly labelled preparations of tRNA from uninfected, $[^{14}C]$, and pseudorabies virus infected, $[^{3}H]$, cells - Fig.32 - shows that the $[^{3}H]$ and $[^{14}C]$ patterns are completely superimposable. The 2 main components which elute at 130 mM and 430 mM-ammonium formate are present in the same proportion in control and pseudorabies virus infected preparations. However, it is questionable whether these components represent aminoacyl-oligonucleotides produced from 2 different species of alanyl-tRNA. Comp. I which elutes at 130 mM-ammonium formate is probably the terminal oligonucleotide from one species. However, the component eluting

Figure 32.

Comparison of Alanyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography of T_1RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with [^{14}C] alanine (non-infected) and [^{3}H] alanine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1RNase digestion products of 500 µg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate buffer, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ, for salt concentration and for total radioactivity associated with each isotope.

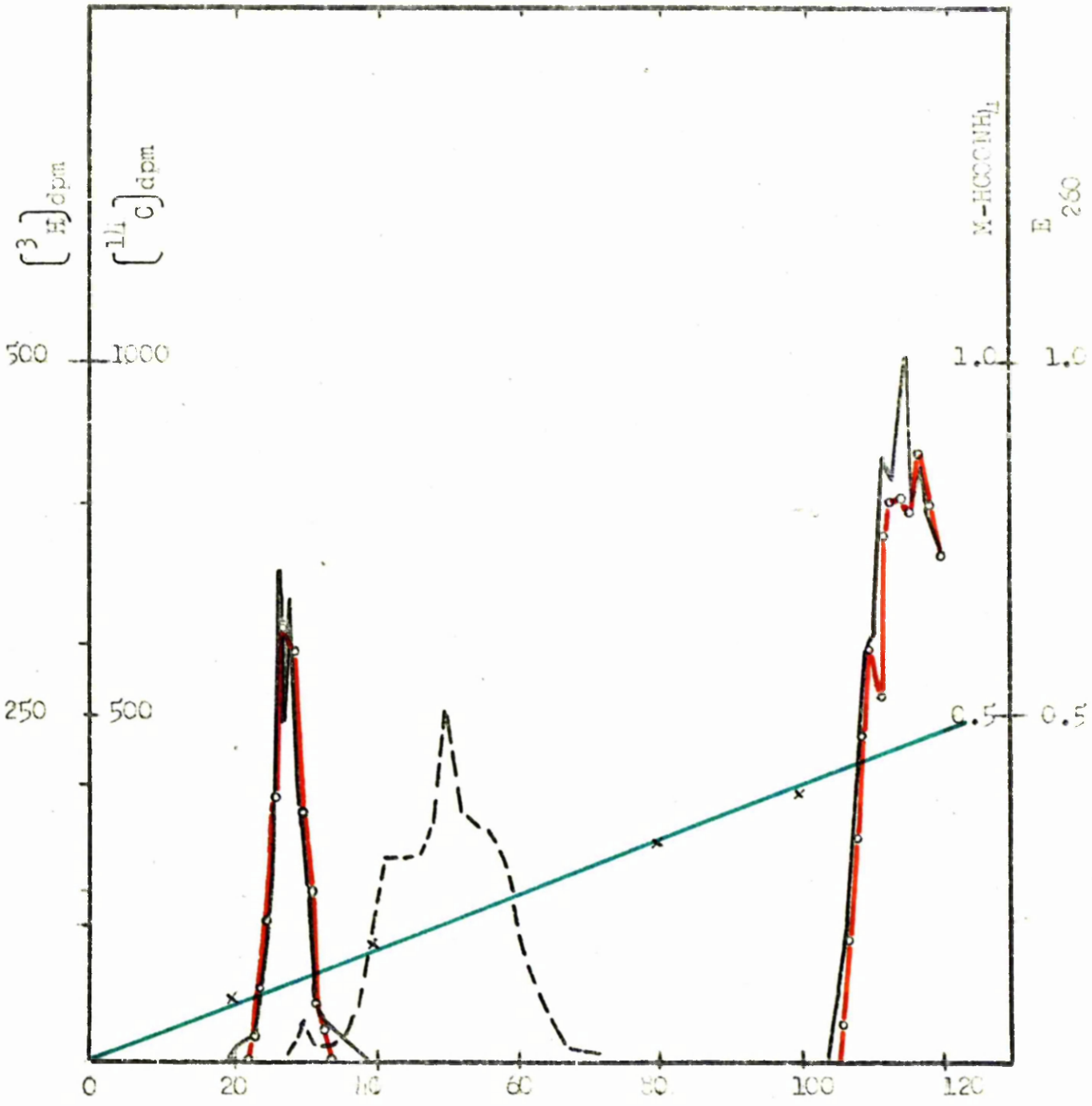
- - - E_{260}

——— [^{3}H] disintegrations/min. /2 ml. of fraction.

_____ [^{14}C] disintegrations/min. /2 ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 32.


$[^{3}H]$dpm
$[^{14}C]$dpm
M-HCOONH4
E 260
500
1000
1.0
1.0
250
500
0.5
0.5
0
20
40
60
80
100
120
Fraction No. (3 ml)

at 430 mM might possibly be an artefact attributable to incomplete digestion of the alanyl-tRNA or to aggregation of alanyl-oligonucleotides. In other systems the nearest guanosine residue to the 3'OH end of alanyl-tRNA occurs at a substantial distance from the amino acid. It is, therefore, quite possible that the fragment eluting at 430 mM-ammonium formate is not an artefact but a long aminoacyl-oligonucleotide.

(c) MAK Column Chromatography

This method makes use of the observations that individual tRNAs often elute from MAK columns at slightly different but characteristic ionic strengths, the actual elution position depending on the molecular size, G+C content and hydrogen bonding (Mandell & Hershey, 1960; Sueoka & Cheng, 1962), of a specific aminoacyl-tRNA. Aminoacyl-tRNAs, chosen from theoretical considerations, from uninfected and pseudorabies virus infected tRNA populations were compared directly on the same column by charging sRNAs prepared in parallel from these sources each with an amino acid labelled with a different

isotope, $[^3H]$ or $[^{14}C]$. Elution was effected by increasing linear sodium chloride gradients and the fractions collected assayed for the presence of both isotopes.

Lysyl-tRNA

Shown in Fig. 33 is the elution profile of non-infected cell and pseudorabies virus infected cell lysyl-tRNA prepared from cells grown in a high serum system, the label of the non-infected cell preparation being $[^3H]$ and that of the virus infected cell preparation being $[^{14}C]$. The patterns are practically superimposable, revealing 2 components present in almost equal proportions. There is, therefore, no difference in the populations which is resolved by MAK column chromatography. However, the number of peaks described probably represents a minimum estimate of the individual lysyl-tRNAs because of the limited nature of MAK column resolution.

Arginyl-tRNA

MAK column chromatography of a mixedly labelled population of arginyl-tRNA prepared from uninfected and pseudorabies virus infected BHK21/13 cells, previously maintained under high serum conditions, routinely gave a

Figure 33.

Comparison of Lysyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography on MAK.

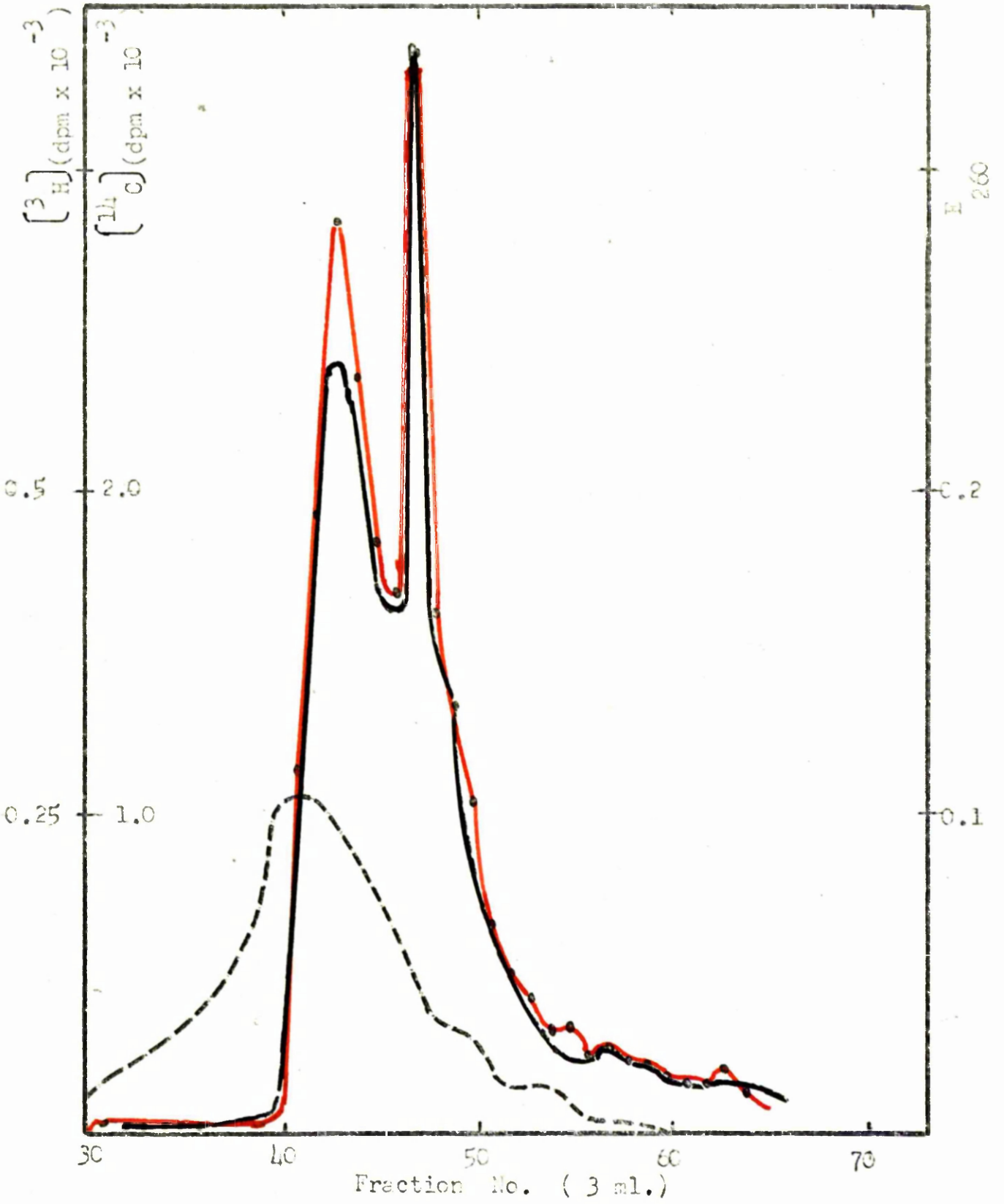
sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with [^{3}H] lysine (non-infected) and [^{14}C] lysine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and a total of 550 µg. of the mixture, in 25 ml., co-chromatographed at 16° on a 2 x 5 cm. column of MAK. A linear gradient of NaCl, buffered to pH 6.25 with NaH_2PO_4/Na_2HPO_4 (50 mM) was used for elution. 3 ml. fractions were collected and immediately placed at 4°. Each fraction was assayed for extinction at 260 mµ and for the trichloracetic acid insoluble radioactivity associated with each isotope.

– – – E_{260}

——— [^{3}H] disintegrations/min./fraction.

——— [^{14}C] disintegrations/min./fraction.

Figure 33.


$[^3H](dpm \times 10^{-3})$
$[^{14}C](dpm \times 10^{-3})$
A_{260}
0.5
2.0
0.2
0.25
1.0
0.1
30
40
50
60
70
Fraction No. (3 ml.)

pattern similar to that shown in Fig. 34. In this case, the $[^{14}C]$ label is attached to tRNA prepared from virus infected cells and the $[^{3}H]$ to that from uninfected cells. Comparison of the elution pattern obtained from the non-infected preparation with that from the pseudorabies virus infected preparation shows no major differences. In both cases the populations are not resolved into distinct peaks, the counts being spread over a wide range of tubes, but are to some extent separated from the main bulk of tRNA as represented by the absorbance at 260 mμ. The two patterns are not completely superimposable in the case illustrated since the control preparations shows a slight shoulder which is not present in the infected preparation. This is, however, probably an artefact and not of any significance. The results, therefore, indicate that no major differences exist between the populations of arginyl-tRNA in BHK21/13 cells and pseudorabies virus infected BHK21/13 cells which can be detected by MAK column chromatography under the conditions used.

Patterns identical to that described above were obtained

Figure 34.

Comparison of Arginyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography on MAK.

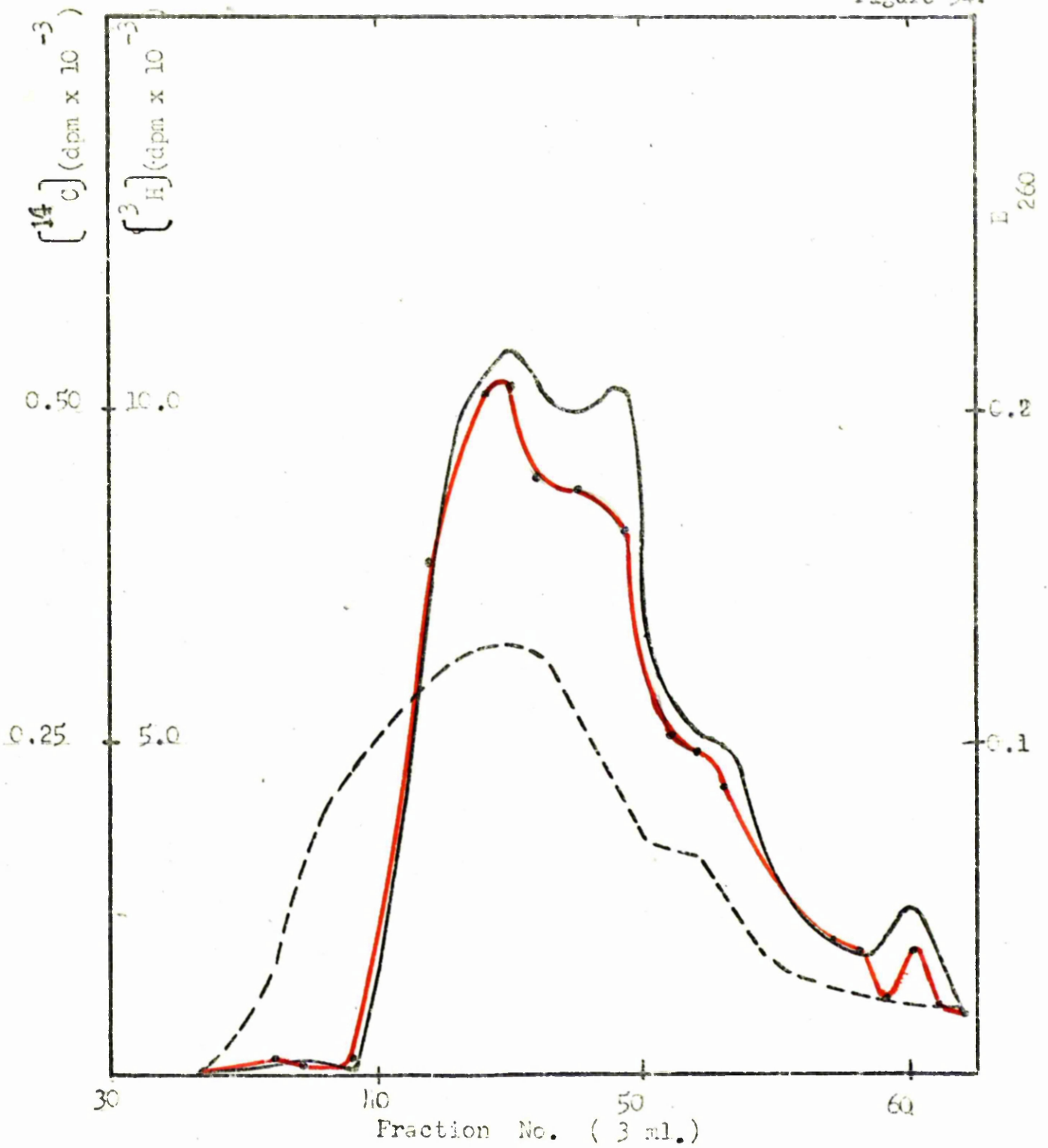
sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with [^{3}H] arginine (non-infected) and [^{14}C] arginine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and a total of 400 µg. of the mixture, in 20 ml., co-chromatographed at 16° on a 2 x 5 cm. column of MAK. A linear gradient of NaCl, buffered to pH 6.25 with NaH_2PO_4/Na_2HPO_4 (50mM) was used for elution. 3 ml. fractions were collected and immediately placed at 4°. Each fraction was assayed for extinction at 260 mµ and for the trichloracetic acid insoluble radioactivity associated with each isotope.

– – – E_{260}

—— [^{3}H] disintegrations/min./fraction.

—— [^{14}C] disintegrations/min./fraction.

Figure 34.


[14C] (dpm x 10-3)
[3H] (dpm x 10-3)
E 260
0.50
10.0
0.2
0.25
5.0
0.1
30
40
50
60
Fraction No. (3 ml.)

on MAK column chromatography of mixedly labelled tRNA preparations even if cells, uninfected or pseudorabies virus infected, in which metabolism had been depressed by deprivation of serum were used as starting material. Chromatography was effected both on a mixture of [^{14}C] arginyl-uninfected tRNA and [^{3}H] arginyl-virus infected tRNA and on a mixture of [^{3}H] arginyl-uninfected tRNA and [^{14}C] arginyl-virus infected tRNA. Therefore, even under conditions in which it might be assumed that the probability of observing differences in the tRNA populations between uninfected and infected cells were maximal, no such alterations were observed.

(d) Reverse Phase Chromatography

Aminoacyl-tRNAs which differ in linear base sequence from one another are known to be separable into specific species on the basis of the charge they carry, using the technique of ion exchange chromatography (Ofengand, Dieckmann & Berg, 1961). Individual species can also be separated by partition between aqueous and organic phases since the coefficient of partition is dependent on the species in question (Khym, 1966). Resolution of the tRNA species

by Reverse phase (type II) chromatography, as developed by Weiss & Kelmers (1967), depends upon a combination of these properties, the partition being between Freon 214 and water and the ion exchange material consisting of aliquat 336 absorbed to Chromosorb W.

Mixedly-labelled preparations, prepared by charging sRNAs extracted from cells before and after pseudorabies virus infection each with an amino acid labelled with a different isotope, were compared directly on the same column. Elution was effected at room temperature by increasing sodium chloride concentration gradients, containing magnesium ions to enhance the resolution, and the fractions collected assayed for the presence of both isotopes.

Unlabelled Aminoacyl-tRNA

Figure 35 shows a typical elution pattern of absorbance at 260 mμ obtained by Reverse phase chromatography of such a preparation. The functioning of the column is clearly satisfactory since it resolves the material into 3 fractions and obviously separates the tRNA (2) from material eluting with the void volume (1), presumably

Figure 35.

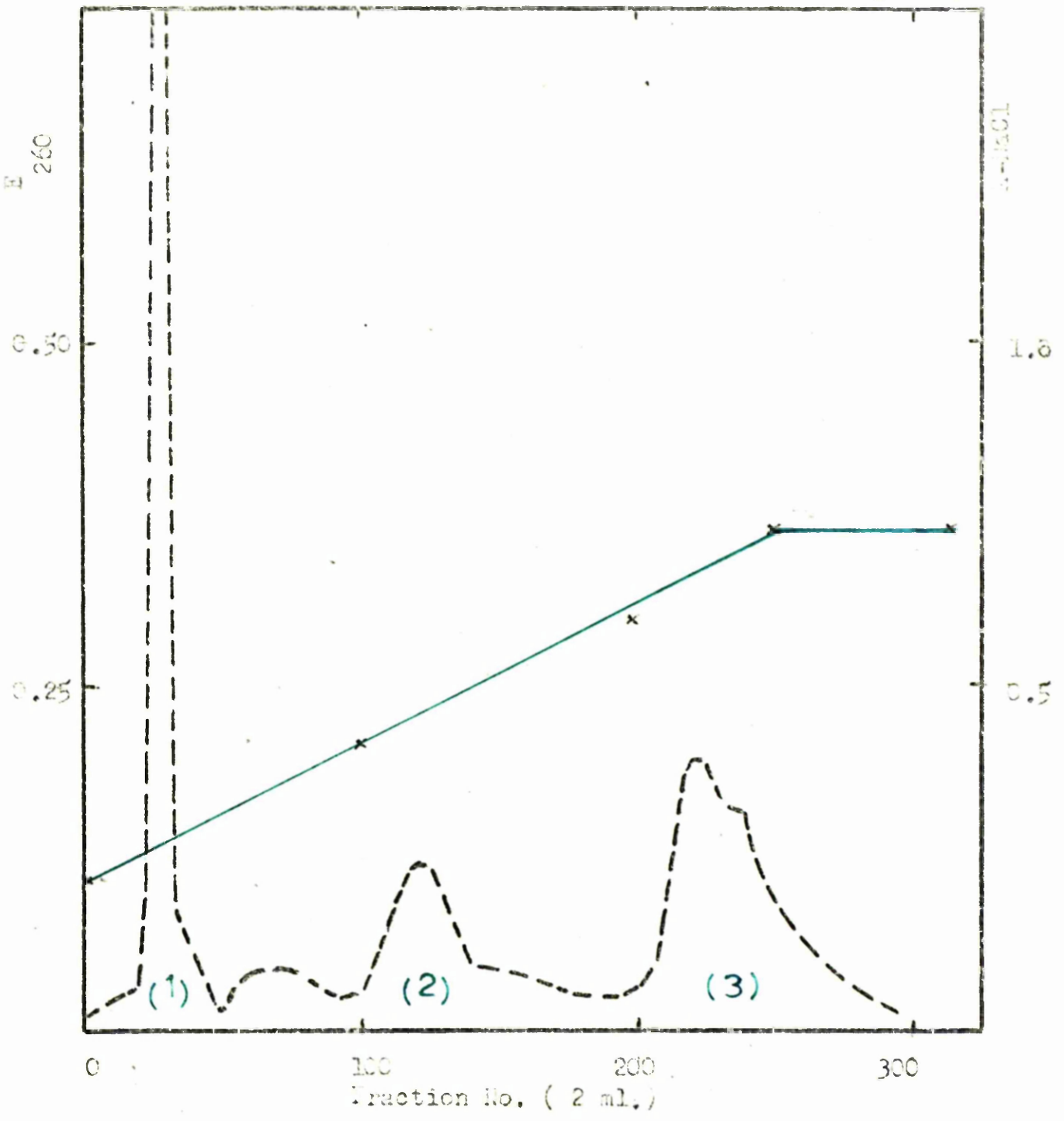
Elution Pattern Obtained By Chromatography of an sRNA Preparation on a Reverse Phase (Type II) Column.

1 mg. of an unlabelled preparation of aminoacyl-tRNA was chromatographed at 16° on an 0.6 x 180 cm. Reverse phase column. A linear gradient of NaCl, buffered to pH 4.5 with sodium acetate (10mM) and containing $MgCl_2$ (10mM) was used for elution. 2 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ. and for salt concentration.

– – – E_{260}

——— NaCl concentration (expressed as molarity).

Figure 35.



degradation products, and from higher molecular weight RNA which elutes at 0.74 M-sodium chloride concentration and is probably ribosomal RNA.

Threonyl-tRNA

Figure 36 shows the elution pattern obtained using Reverse phase (type II) chromatography of a mixedly labelled threonyl-tRNA preparation. In this case, the $[^{14}C]$ is derived from uninfected BHK21/13 cell tRNA and the $[^{3}H]$ label from pseudorabies virus infected BHK21/13 cell tRNA. The pattern consists of 2 major peaks of radioactive material with the suggestion of a third minor peak at tube 131.

The peak of $[^{3}H]$ labelled material appearing at tube 137 is probably introduced by calculation since a slight underestimate of $[^{14}C]$ disintegrations is immediately converted by the method used to an excess of $[^{3}H]$ disintegrations. All 3 genuine peaks contain both $[^{3}H]$ and $[^{14}C]$ labelled material and the distribution of one label over the fractions is exactly mirrored in the distribution of the other.

The peaks of radioactive material obtained, although

Figure 36.

Comparison of Threonyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography on a Reverse Phase (type II) System.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6 hr. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^{14}C]$ threonine (non-infected) and $[^{3}H]$ threonine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and a total of 1.5 mg. of the mixture co-chromatographed at 16° on a 0.6 x 180 cm. Reverse phase column. A linear gradient of NaCl, buffered to pH 4.5 with sodium acetate (10mM) and containing $MgCl_2$ (10mM) was used for elution. 2 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ, salt concentration and in the tRNA region for trichloracetic acid insoluble radioactivity associated with each isotope.

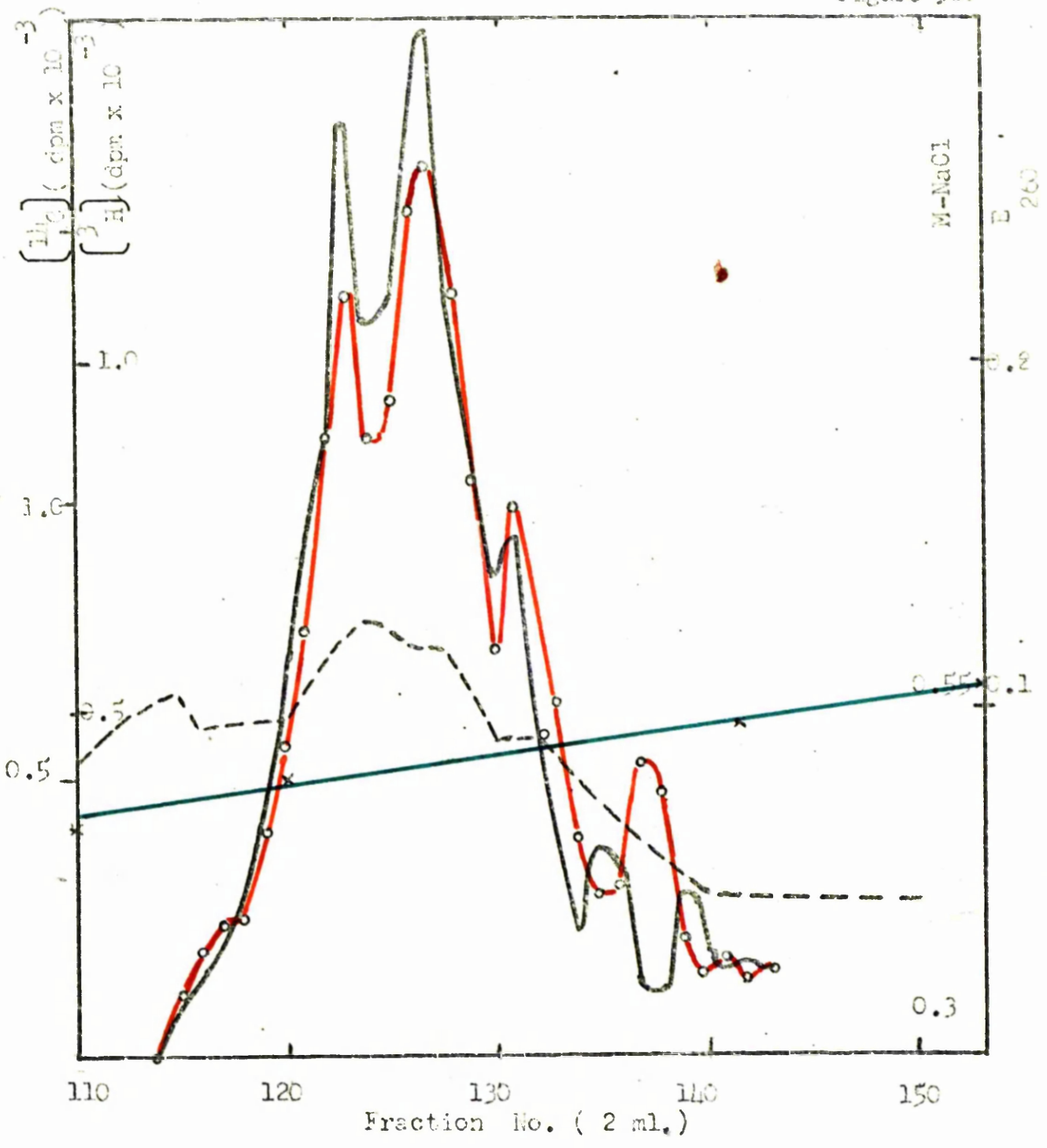
– – – E_{260}

——— $[^{3}H]$ disintegrations/min./fraction.

——— $[^{14}C]$ disintegrations/min./fraction.

——— NaCl concentration (expressed as molarity).

Figure 36.


$[^{14}C]$ (dpm x 10^{-3})
$[^{3}H]$ (dpm x 10^{-3})
M-NaCl
E_{260}
1.5
1.0
0.5
0.2
0.1
0.3
110
120
130
140
150
Fraction No. (2 ml.)

separated from the bulk of the tRNA present, as measured by absorbance at 260 mμ, are not sufficiently resolved from one another to merit a meaningful estimation of the proportion of radioactive material in each peak. However, the results indicate that threonyl-tRNA extracted from pseudorabies virus infected cells does not contain any component resolvable by the method used which is not present in uninfected cells. Taking the simplest view, the results also indicate that there exists at least two and perhaps three distinct species of tRNA.

Seryl-tRNA

When eluting, by increasing salt concentration, from a Reverse phase (type II) column, a mixture of $[^{14}C]$ seryl-tRNA from uninfected cells and $[^{3}H]$ seryl-tRNA from pseudorabies virus infected cells gives the profile shown in Fig. 37. Radioactively labelled material is restricted to a relatively small area of the tRNA elution spectrum, as measured by the spread of absorbance at 260 mμ and so satisfactory resolution has occurred.

There appear to be 4 major peaks of radioactive

Figure 37.

Comparison of Seryl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography on a Reverse Phase (type II) System.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6 hr. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^{14}C]$ serine (non-infected) and $[^{3}H]$ serine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and a total of 1.5 mg. of the mixture co-chromatographed at 16° on a 0.6 x 180 cm. Reverse phase column. A linear gradient of NaCl, buffered to pH 4.5 with sodium acetate (10mM) and containing $MgCl_2$ (10mM) was used for elution. 1 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ. salt concentration and in the tRNA region for trichloracetic acid insoluble radioactivity associated with each isotope.

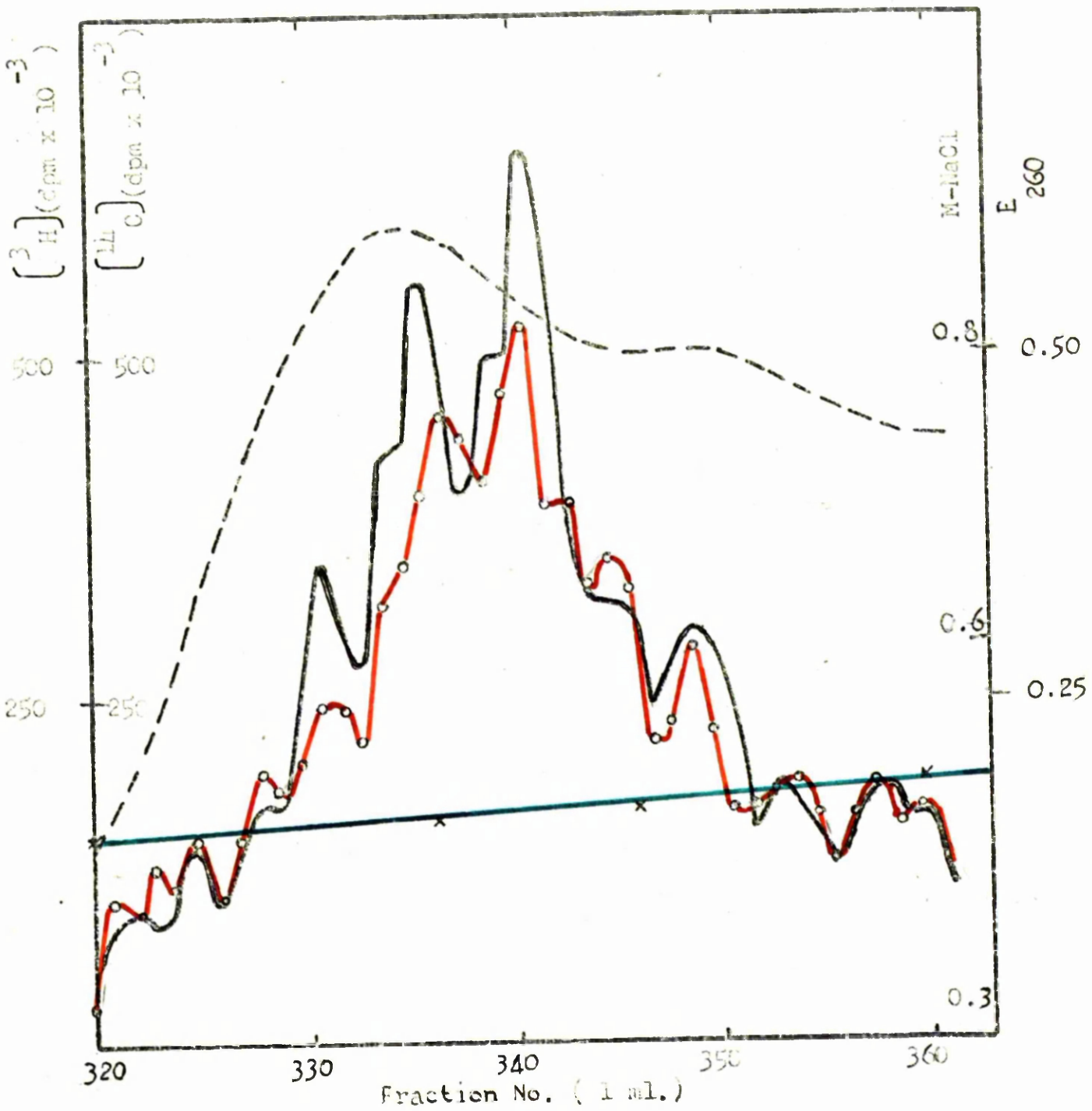
– – – E_{260}

——— $[^{3}H]$ disintegrations/min./fraction.

——— $[^{14}C]$ disintegrations/min./fraction.

——— NaCl concentration (expressed as molarity).

Figure 37.


$[^3H](dpm \times 10^{-3})$
$[^{14}C](dpm \times 10^{-3})$
500
500
250
250
M-NaCl
E_{260}
0.8
0.50
0.6
0.25
0.3
320
330
340
350
360
Fraction No. (1 ml.)

material - both [^{14}C] and [^{3}H] - centred at tubes 331, 340 and 349 with also the suggestion of a fifth peak at tube 345. Subsequently, these peaks will be termed components, I, II, III, V and IV respectively.

The background corrected [^{3}H] and [^{14}C] disintegrations in the 5 peaks are -

	I	II	III	IV	V
[^{14}C]	1150	2300	3000	900	1275
[^{3}H]	925	1925	2550	900	900

and so the ratio of total [^{3}H] radioactivity in each component to that in component I is (II/I) 2.03:1; (III/I) 2.64:1; (IV/I) 0.8:1 and (V/I)1.1:1. The corresponding [^{14}C] ratios have values 2.14:1; 2.83:1; 1:1 and 1:1.

Therefore, although the elution patterns of uninfected and pseudorabies virus infected cell seryl-tRNA, as represented by [^{14}C] and [^{3}H] disintegrations, do not appear directly superimposable, the ratio of material in the components appears identical within the limits of experimental error with the possible exception of component IV which may possibly be present in slightly higher

proportions in the infected preparation than in the uninfected. The significance of this observation is doubtful in view of the relatively low number of disintegrations involved. The probability is that no 'new' infected tRNA components, resolvable by this method, are present although it should be noted that, because of the low number of disintegrations collected, components constituting less than 5% of the major component would not be detected. In addition, the elution pattern indicates that BHK21/13 cells contain possibly 5 seryl-tRNA species as a minimum, assuming that each component resolved represents a separate tRNA species.

Arginyl-tRNA

Illustrated in Fig. 38 is the elution profile obtained on Reverse phase (type II) column chromatography of a preparation of [^{3}H] labelled arginyl-tRNA from pseudorabies virus infected cells mixed with one of [^{14}C] labelled arginyl-tRNA from uninfected cells. In this case, the resolution of the column appears to be much poorer as the radioactive material is widely distributed over the gradient, is not well separated from other tRNA species

Figure 38.

Comparison of Arginyl-tRNA from Non-infected and Pseudorabies Virus Infected BHK21/13 Cells by Co-chromatography on a Reverse Phase (type II) System.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6 hr. P.I. at a multiplicity of exposure of 20 PFU per cell with pseudorabies virus. The preparations were separately charged with $[^{14}C]$ arginine (non-infected) and $[^{3}H]$ arginine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and a total of 1.5 mg. of the mixture co-chromatographed at 16° on a 0.6 x 180 cm. Reverse phase column. A linear gradient of NaCl, buffered to pH 4.5 with sodium acetate (10mM) and containing $MgCl_2$ (10mM) was used for elution. 2 ml. fractions were collected and immediately placed at 4°. Each fraction was monitored for extinction at 260 mμ., salt concentration and in the tRNA region for trichloracetic acid insoluble radioactivity associated with each isotope.

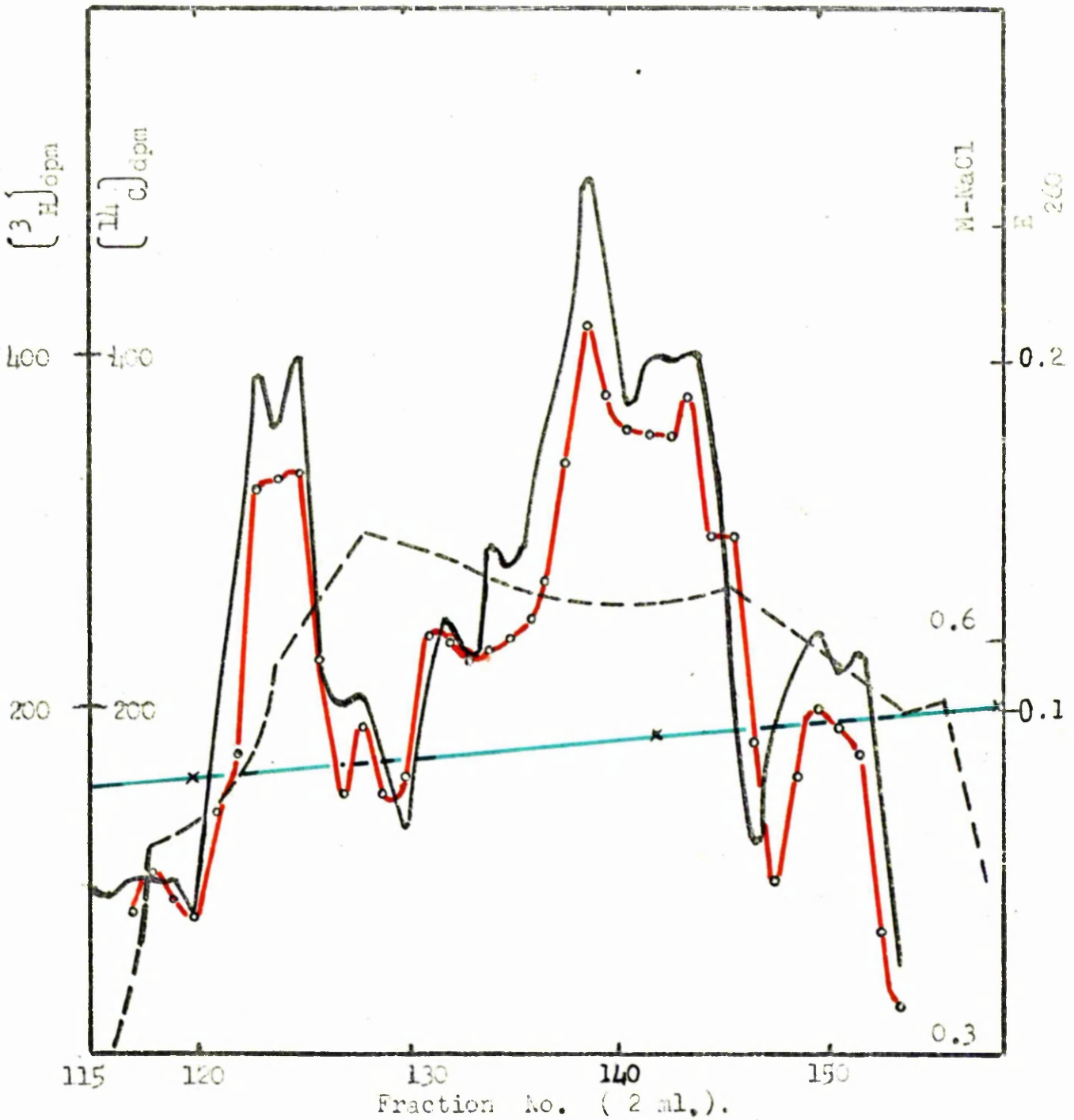
- - - E_{260}

——— $[^{3}H]$ disintegrations/min./fraction.

—— $[^{14}C]$ disintegrations/min./fraction.

——— NaCl concentration (expressed as molarity).

Figure 38.


$[^3H]_{dpm}$
$[^{14}C]_{dpm}$
M-NaCl
E 260
400
200
0.2
0.6
0.1
0.3
115
120
130
140
150
Fraction No. (2 ml.).

(detected as U. V. absorbing material) and since the profiles themselves do not appear smooth. The number of disintegrations collected per minute per fraction is also low and it is obviously not justifiable to make any quantitative calculations. The elution patterns of $[^{14}C]$ and $[^{3}H]$ labelled material appear to be almost superimposable with one another, both comprising 2 relatively sharp peaks at tubes 125 and 152 and one broad peak centred at tube 140. These results indicate that (a) assuming that each peak represents a separate tRNA species, the minimum number of arginyl-tRNA species in uninfected BHK21/13 cells is 3 and is not an artefact of extraction, and (b) no differences resolvable by this method exist between preparations of arginyl-tRNA from uninfected and pseudorabies virus infected BHK21/13 cells. However, it should again be emphasised that the number of disintegrations collected per fraction is so low that minority species in either uninfected or infected preparations might remain undetected.

DISCUSSION

Examination of nearest neighbour sequence analysis data suggested very pronounced differences in the base sequence of the DNA of herpes simplex virus and of pseudorabies virus from that of mammalian cells (Subak-Sharpe, Bürk, Crawford, Morrison, Hay & Keir, 1966). From theoretical considerations, it was postulated that the amino acid composition of proteins synthesised pre and post infection (with either of the viruses mentioned) would differ substantially and that both viruses might require, and therefore specify, certain tRNAs - particularly those recognizing CpG containing codons (Subak-Sharpe, Shepherd & Hay, 1966).

Supporting evidence for this hypothesis was obtained using herpes simplex virus infection of BHK21/13 cells and is summarised below. 4s RNA from herpes simplex virus infected, but not from uninfected cells, was known to participate in molecular hybridisation with herpes virus DNA (Subak-Sharpe & Hay, 1965). These DNA-RNA hybridisation experiments gave a clear indication of virus specific RNA molecules with properties closely resembling tRNA. Data obtained by MAK column chromatography suggested the presence in infected cells of arginyl specific tRNAs not

present in the uninfected cells (Subak-Sharpe, Shepherd & Hay, 1966).

Elucidation of the effects of pseudorabies virus infection were therefore undertaken. Firstly, data concerning alterations in the amino acid content of proteins newly synthesised in uninfected and pseudorabies infected BHK21/13 cells were presented. Indications of increased arginine incorporation after pseudorabies infection, with concomitant decrease in lysine incorporation, were obtained. The experimentally determined values, referred to control values of unity, for arg./lys., val./lys. and arg./val. incorporation in pseudorabies virus infected cells, 10 hours post infection, were respectively 3.2, 0.6 and 1.6 (Fig. 8). By assuming that protein synthesis in pseudorabies virus infected cells at this time is completely specified by the virus DNA, theoretical estimates of these incorporation ratios were also made as follows. The expected frequencies of nucleotide doublets in pseudorabies virus DNA and host cell DNA, based solely on their G+C content, were derived as described in Subak-Sharpe, Shepherd & Hay (1966). The amino acid frequencies for arginine, lysine and valine in proteins specified from both these DNAs were then transposed from the

frequencies, by reference to the known genetic code, and the incorporation ratios for arg./lys., val./lys. and arg./val. in both sources calculated. After assigning values of unity to the ratios obtained for cellular specified proteins and suitably correcting the virus data, values of 5.7, 0.41 and 2.4, respectively, were obtained for arg./lys., lys./val. and arg./val. These agree reasonably well with the experimentally obtained values quoted above.

Investigations of the sRNA in the cytoplasmic fractions of BHK21/13 cells pre and post infection with pseudorabies virus, were reported. Cytoplasmic cell fractions were used as sRNA sources since it appears that viral proteins are synthesised in the cytoplasm even though mature virus particles are formed in the nucleus (Ben Porat, Shimono & Kaplan, 1969). The 4s RNA from infected cells was investigated using the molecular hybridisation technique of Gillespie & Spiegelman (1965) in an attempt to demonstrate, and perhaps quantitate, sequence homology. Preparations derived from non-infected and pseudorabies virus infected cells were tested against both cellular DNA and DNA from purified pseudorabies virus particles.

No hybridisation was observed between pseudorabies virus

DNA and sRNA from uninfected cells. It was, therefore, concluded that the viral DNA was not contaminated with cellular DNA and did not contain short sequences fortuitiously homologous with 4s RNA from uninfected cells. The data obtained by saturation tests confirmed the existence in BHK21/13 cells of a low molecular weight - RNA homologous with 0.01% of the cellular DNA (c.f. a value of 0.023% in E. coli (Giacomoni & Spiegelman, 1962) and one of 0.015% in Drosophila melanogaster (Ritossa, Atwood & Spiegelman, 1966)). In pseudorabies virus infected cells there was, in addition to 4s RNA hybridisable with cellular DNA (i.e. host specified 4s RNA), a low molecular weight RNA which hybridised with viral DNA i.e. was virus specified. This species occupied 0.15% of the viral genome and was not 5s. It could not, however, be unequivocally identified as tRNA although it possessed the characteristic molecular size (on Sephadex-G100), contained methylated bases and showed no trace of randomly degraded radioactively labelled mRNA.

Under the assumption that the species in question was tRNA, a further degree of quantitation can be considered. Reference to the data previously discussed (refer to page 158) shows that 20 μg. of viral DNA hybridised at saturation with 250 counts/minute of

sRNA derived from pseudorabies virus infected cells, labelled between 3 and 8 hours post infection. DNA specifying sRNA comprised 0.15% of the viral genome, assuming the specific activity of the genuinely virus coded sRNA to be uniform and equal to that experimentally determined for the infected cell sRNA preparation. Given an average molecular weight of 25,000 for tRNA and 7×10^7 for viral DNA (Russell & Crawford, 1963) and assuming that saturation had been achieved, it was calculated that 4 tRNA molecules were specified by the virus. The value obtained was obviously an estimate of the maximum number of virus coded species since the value assumed for the specific activity must be an underestimate (due to the pre-existing cellular tRNA). The data obtained did not suffice to permit calculation of the original specific activity of the virus coded sRNA. Thus, an unequivocal estimate of the number of tRNAs specified per viral DNA molecule has not been achieved. Extension of the investigation to involve competition experiments would have provided a better estimate of this value but even so it would have been necessary to assume that viral infection had not altered the spectrum of host tRNAs synthesised.

In general, the validity of detection and quantitation of

sequence homology has many inherent sources of error. Estimates may either be invalidated by failure to reach DNA saturating levels of RNA or by the presence of a minor impurity, which occupies a much larger proportion of the DNA, in the hybrid structure. The magnitude of the error depends on the length of the DNA genome homologous with the RNA under investigation.

The experimental results obtained were open to the criticism that the duration of the hybridisation period for attainment of DNA saturation was not rigidly determined. But the experimental results were not, to any great extent, open to criticism concerning possible contamination with degraded mRNA. Accuracy of the quantitative values quoted was not high, partly due to questionable saturation, but also because the level of radioactive material detected in the hybrid material was so low. In order to raise the level of hybrid associated radioactivity to a more significant value, it would obviously be necessary to use more viral DNA in the assay system. No experiments using higher DNA levels were, however, attempted since the preparation of such large quantities of viral DNA presented technical difficulties (one virus particle contains 10^{-16} g. of DNA).

The results were, however, repeatable and permitted the conclusion that pseudorabies virus specified certain species of low molecular weight RNA - which appeared to be tRNA. Definite identification of the low molecular weight component was not made and it would obviously be very interesting to extend the investigations to include studies on the hybridisation of viral DNA with amino acid labelled aminoacyl-tRNA, using the system developed by Bonner, Kung & Bekhor (1967).

Differences between the populations of certain aminoacyl-tRNAs in non-infected and pseudorabies virus infected cells were investigated using combinations of three different chromatographic procedures to examine differentially labelled mixed populations of tRNA from non-infected and infected cells. The uninfected tRNA carried a tritium labelled amino acid, say, and the infected tRNA the same amino acid but labelled with $[^{14}C]$.

The three methods used were as follows.

(a) MAK column chromatography which separates according to hydrogen bonding and G+C content (Mandell & Hershey, 1960).

(b) DEAE cellulose chromatography of T_1 RNase digests which detect any species of tRNA, the 3' terminal sequence

of which differs with respect to the position of the guanosine residue nearest to that end (Ishida & Miura, 1965).

(c) Reverse phase chromatography which separates on the basis of ion exchange and partition between organic and aqueous phases (Weiss & Kelmers, 1967).

At present, arginyl-tRNA and lysyl-tRNA have been examined by MAK column chromatography, arginyl-tRNA, lysyl-tRNA, seryl-tRNA and alanyl-tRNA by DEAE cellulose chromatography of T_1 RNase digests and threonyl-tRNA, seryl-tRNA and arginyl-tRNA by Reverse phase chromatography. In all cases, the control and infected patterns were almost superimposable and neither indicated the presence of any new species of tRNA nor any alteration of the balance of the host population. The results described were obtained both using actively growing and "stationary" (low serum) cells although detection efficiency was assumed to be higher in cells maintained in serum deficient medium.

These results, taken at face value imply, that the tRNA populations for arginine, serine, threonine, alanine and lysine remained unaltered by pseudorabies virus infection. By examination of those cases in which multiple iso-accepting tRNAs were detected in uninfected cells, it was also concluded

that pseudorabies virus infection did not lead to an alteration in the relative proportions of these species. However, the failure to detect species of tRNA not observed in uninfected cells does not necessarily preclude their occurrence. There are certain inherent limitations in the techniques used, any one of which could have masked the presence of a 'new' or 'modified' tRNA. The limitations envisaged are enumerated and discussed below.

1. The aminoacyl derivative may never have been labelled with radioactive amino acid and so remained undetected in a system using the altered distribution of the aminoacyl moeity as criterion for comparisons. The absence of labelled material may have been due to any of the following reasons.

(a) The in vivo loaded pseudorabies virus specified or modified aminoacyl-tRNA, as extracted, possessed an ester bond which was resistant to the amino acid stripping technique employed. In this case, radioactive label could only be introduced in exchange reactions - an unlikely possibility.

(b) Configurational changes occurred in the tRNA during extraction which prevented or slowed amino acid esterification.

(c) Malfunction occurred *in vitro* of an aminoacyl-tRNA transferase the *in vivo* activity of which was to catalyse the attachment of the appropriate amino acid to the 'new' or 'modified' tRNA.

2. The aminoacyl derivative of the 'new' or 'modified' tRNA may have been labelled but the ester bond, although maintained at the pH believed optimal for its stability (5.5), did not survive the subsequent chromatographic procedures.

3. The chromatographic methods used may have failed to separate the 'new' or 'modified' aminoacyl-tRNA species from the corresponding pre-existing host aminoacyl-tRNAs.

4. The chromatographic methods used may have resulted in the elution of the 'new' or 'modified' aminoacyl-tRNA species in a position outwith that normally associated with aminoacyl-tRNAs i.e. in a region not examined for radioactivity.

5. The tRNA species in question although, perhaps, present locally in high concentration may have represented such a small percentage of the total cellular population as to have been undetectable after destruction of cell compartmentation.

Each of these alternatives is now discussed.

1(a) In view of reports on the lability of aminoacyl-tRNA ester bonds to mild alkaline hydrolysis, (Gatica, Allende, Mora, Allende & Medina, 1966) the in vitro unloading conditions used should be more than adequate to hydrolyse all the aminoacyl-bonds. It is, however, known that in certain cases the bond stability is altered. For instance, N-acyl amino acid derivatives are esterified to tRNA by bonds which are more stable at high pH than are the corresponding bonds between amino acid and tRNA. Also, $tRNA^{leu.}$ can be isolated attached, in the 3'OH or 2'OH position, to a 'protector' substance by an alkali resistant bond (Yegian & Stent, 1969b). The occurrence of such isolated cases does not seem sufficient reason to regard this alternative as plausible, especially, since a 'protector' type substance would be enzymically removed during in vitro aminoacylation.

1(b) The alternative suggestion of configurational alterations in the tRNA is also unlikely. Initially such an explanation appeared highly feasible since the conditions during tRNA extraction (EDTA and phenol) could be correlated with those

documented as causing 'denaturation' of tRNA (Lindahl et al., 1967; Gartland & Sueoka, 1966). 'Denaturation' is the term applied to the conversion of one configurational form of tRNA which is capable of accepting amino acids to another which no longer does so. It has only been observed in respect to certain types of tRNA, the identity of which depended on the organism and on the amino acid involved. A 'new' species of tRNA might therefore be present but in (a) a form completely incapable of undergoing enzymic acylation or (b) a form incapable of undergoing enzymic acylation at a rate comparable with that of the corresponding cellular tRNA species (Yegian & Stent, 1969a). However, no apparent differences resulted from renaturation by site binding of Mg^{2+} (Lindahl, Adams & Fresco, 1966) and it may, therefore, be concluded that such an explanation is invalid in the system used. It should, however, be noted that leucyl-tRNA F (an expression of T_2 phage function) once unloaded by exposure to highly alkaline conditions cannot be reloaded due, it is thought, to degradation of the tRNA (Kano-Sueoka & Sueoka, 1966). There are, also, reports of a species of $tRNA^{isoleu.}$ which cannot accept isoleucine when in a reduced state (Yegian & Stent, 1969(a) (b)).

It may, therefore, be that the tRNA configuration could be altered in a completely novel, so far undefined, way.

Alternative 1(c) which implicates the transferase enzyme, cannot be so summarily rejected. It can be envisaged to occur in at least two ways, one of which necessitates the production of a virus coded aminoacyl-tRNA transferase.

(i) By involving enzyme malfunction, alteration in the properties of the transferase must, by and large, be postulated - (provided that the unlikely possibility of a very labile as yet undetected host transferase is not considered). In other words, the existence in vivo of a virus coded enzyme or a virus modified host enzyme is assumed. Its function would be to acylate a tRNA (or more than one) recognizing CpG containing codons and its enzymic properties must differ markedly from the corresponding host transferase. Perhaps, the difference can be assigned in one of the following ways. Firstly, the infected cell enzyme may be so labile as to be inactivated by the extraction procedures. Conversely, the ionic conditions normally employed for in vitro

acylation may not be optimal for the infected cell enzyme, perhaps as regards the relative concentrations of magnesium ions and ATP (Novelli, 1967) or as regards monovalent ions such as ammonium (Yu & Hirsh, 1967; Loftfield & Eigner, 1967). The latter have been reported to enhance manyfold the activity of transferases which previously appeared of very minor consequence (Yu, 1966). Thirdly, the infected cell enzyme may have a Km which differs sufficiently from the corresponding host enzyme as to require an entirely different amino acid concentration for optimal activity. In this respect, it should be noted that a mutant strain of E. coli has been shown to possess glycyl, histidinyl and phenylalanyl transferases the Km values for which differ from those in wild type cells (Neidhardt, 1966).

(ii) There are, however, other explanations which do not require the formation of a new aminoacyl-tRNA transferase. High concentrations of sodium chloride in the acylation mixtures have been shown to cause a transferase, previously capable of catalysing homologous and heterologous loading, to catalyse only homologous loading (Peterkofsky,

Gee & Jesensky, 1966). Perhaps, therefore, the ionic conditions provided were not those required for the host transferase to attach an amino acid to the 'new' or 'modified' tRNA species i. e. to partake in heterologous loading. The efficiency of certain acylation reactions has also been reported to be affected by organic solvents (Loftfield & Eigner, 1967). Therefore, in vitro conditions may not be reproducing the ideal phase conditions stabilised in vivo by a particular (membrane-soluble) distribution of the enzyme.

Since it is possible that virus infection may be associated with changes in intracellular pH, ionic conditions or compartmentation which may affect the rate or extent of function of aminoacyl-tRNA transferases, all the reasons just examined provide a logical explanation for the failure to detect any alteration in the tRNA population. Pseudorabies virus infected RK cells become very leaky indeed as compared to normal, (Zemla, Coto & Kaplan, 1967). In addition, such explanations are obviously more attractive since they do not infer the existence of a new enzyme the formation of which might require the participation of the product of the reaction catalysed

by that enzyme. The hypothesis outlined above is also attractive since it can account for the failure to detect any different species of arginyl, seryl, threonyl, lysyl and alanyl-tRNA i. e. all the viral induced aminoacyl-tRNAs are formed with the highest efficiency under conditions identical to the in vivo environment. The in vitro loading conditions, therefore, are considered suboptimal for all the aminoacylation reactions affected by virus infection.

2. The feasibility of the explanation outlined in proposal 2 i. e. unstable aminoacyl ester bonds, cannot be directly established. Therefore it is impossible to draw firm conclusions. Only two indications are available. Firstly, it is known that aminoacyl moeities may be lost at pH 5 and that the half lives reported in different systems for aminoacyl-tRNAs do cover a wide range of values. It could, therefore, be possible that the stability of the ester bond in control arginyl-tRNA say, may be considerably higher than that of 'new' arginyl-tRNA from infected cells. Secondly, all the aminoacyl-tRNAs examined have been shown stable at least for 1 hour at 37° and pH 5. 5 (p. 167). It would, therefore, appear that absence of amino acid label

cannot be completely explained on this basis.

3. The explanation advanced under this heading is a valid one. That is to say, the detection of 'new' or 'modified' aminoacyl-tRNAs may have been precluded by the limited resolving powers of the techniques employed. Using the correct technique, aminoacyl-tRNA molecules which differ in only one or a few bases are clearly separated (Gefter & Russell, 1969; Goodman et al., 1968; Madison, Everett & Kung, 1966; Zachau et al., 1966), whereas in other cases, molecules of very different base sequences are not resolved. For instance, a species of prolyl-tRNA of base sequence homologous with T_4 DNA, isolated from T_4 infected E. coli, was not detected by MAK column chromatography (Weiss et al., 1968). The chromatographic techniques selected were perhaps not the most appropriate for resolving the structural divergencies of the species involved. However, as it is impossible (a) to even deduce whether putative viral induced species, if they exist, are modified versions of host coded tRNAs or are themselves virus coded or (b) to rigorously define the criteria for species separation operating in the chromatographic procedures available, a reasoned selection

cannot be made. Utilization of other chromatographic techniques, selected to give as wide a range of separation characteristics as possible, might provide more definite data.

4. The possibility that virus induced, or even virus modified aminoacyl-tRNA species, might possess such novel structural characteristics as to chromatograph in completely atypical positions must be introduced in view of the gel filtration data presented (p.137). Such an explanation, however, although possibly tenable for MAK column chromatography (where fractions eluting at very high salt concentrations were not rigorously examined), must be eliminated by the results obtained by DEAE cellulose chromatography of T_1 RNase digests. Enzymic digestion would result in oligonucleotide production, no matter the original structure of the RNA.

5. The explanation proposed under this heading cannot be directly examined experimentally nor assessed theoretically. Reference to reported data, shows that no quantitative difficulties were experienced in demonstrating the existence of T_4 phage specified tRNA nor in detecting arginyl-tRNA specified by the related herpes simplex virus. It should,

however be noted that in the amber suppressor system of $tRNA^{tyr.}$ special conditions were introduced to increase the yield of suppressor tRNA to a detectable level.

It may, therefore, be concluded that pseudorabies virus specifies at most 4 tRNA species but that at present the identification of these species has not been possible, probably for technical reasons.

Pseudorabies virus does however appear to effect alterations in the pattern of synthesis of low molecular weight RNAs - 4s and 5s RNA. A progressive decrease in the ratio of 4s/5s RNA synthesised per unit time was detected with time after infection. This was, in the main, attributable to a decreased rate of 4s RNA synthesis although a 1.5-2 fold increase in 5s RNA synthesis was also detected. Investigations described showed that the increased 5s RNA synthesis could not be correlated with that reported in adeno 2 virus infected cells, which possess, late in infection, a large amount (10 times normal) of cytoplasmically located 5s material (Reich et al., 1966). With pseudorabies virus infection, the increase is obviously not of the same magnitude as that described nor does it first appear at the same position in the viral multiplication cycle. Investigations of the intracellular

location of the 5s material in pseudorabies virus infected cells were inconclusive although separations and extractions were carried out at all times at high concentrations of magnesium ions. That is to say, experimental conditions were optimal for normal 5s RNA binding to ribosomes (Siddiqui & Hosokawa, 1968). The increased level of 5s RNA synthesis, if genuine, cannot therefore be simply explained as virus specified "normal" 5s material. Its exact nature and function remain unidentified.

The $[^3H]$ pulse labelling experiments described in this report showed that as early as 2 hours post infection there existed in pseudorabies virus infected cells a species of RNA - termed $4\frac{1}{2}$s RNA - not detectable in the uninfected cells. From its gel filtration characteristics, it was deduced to be either of higher molecular weight or to have a less compact structure than tRNA (4s). Reference to the literature indicated that examples of RNA detected eluting in the $4\frac{1}{2}$s region were diversely identified as

(a) aggregates of 2 or more tRNA molecules (Schleich & Goldstein, 1964; Röschenthaler & Fromageot, 1965; Loehr & Keller, 1968).

(b) tRNA species synthesised after phage infection of E. coli

with T4 and coded by the T4 genome. These species were known to possess abnormal sulphation patterns and to be capable of accepting amino acids. Material in this peak did not appear to migrate to the 4s position even after prolonged exposure to the isotope. Consequently, these species were thought to represent 'mature' tRNAs specified by T4 (Weiss et al., 1968).

(c) tRNA precursors, sometimes termed pre-tRNAs, as detected in Krebs II ascites cells. These species appeared very rapidly in the cytoplasm and were deficient in methylated bases (Burdon, Martin & Lal, 1967). In vivo they were quickly converted to 4s RNA (10 minutes in BHK21/13), while an almost identical conversion was achieved in vitro by exposure of the RNA to conditions known to cause specified configurational changes of tRNAs (Burdon, 1967). In particular, the conditions were known to cause conversion, without alteration of the molecular weight, of an open assymetrical molecule to the more compact (presumably cloverleaf) configuration characteristic of 'mature' tRNA. Monitoring under different ionic conditions confirmed that the alteration observed was mainly a configurational one. Although the

product formed by this technique was still fractionally larger than 'mature' tRNA, the new components were considered to be precursors of tRNA detected prior to methylation, perhaps, with an open configuration or with a slightly longer base sequence than the 'mature' RNA (Burdon & Clason, 1969).

The $4\frac{1}{2}$s material detected in pseudorabies virus infected cells displayed several characteristics in common with the putative tRNA precursors, in that, no methyl groups (derived from [^{14}C] methyl methionine) were detected and that the molecules were short lived in this form and were rapidly converted in vivo to 4s RNA (in contrast to the T_4 phage-coded RNA species). Further in vitro experiments, however, failed to demonstrate interconversion of the species by the renaturation techniques already mentioned (Lindahl, Adams & Fresco, 1966). It was, however, possible to effect a considerable measure of conversion in vitro by incubation of the $4\frac{1}{2}$s material with crude extracts of uninfected (but not infected) cells.

Assuming that the RNA molecules under consideration are similar to those reported in Krebs II ascites cells - i.e. are pre-tRNAs, it is interesting to speculate on the nature of the

causative virus induced metabolic changes. $4\frac{1}{2}$s RNA cannot only be an intermediate peculiar to the synthesis of virus coded tRNA since infected cell extracts are incapable of efficiently converting it to 'mature' virus coded tRNA. Similar reasoning also precludes the identification of all $4\frac{1}{2}$s RNA as a host specified virus modified precursor of a virus induced tRNA. (The presence of very low levels of virus specified or modified tRNA cannot be precluded on the experimental data available). The majority of this species must, however, represent a host cell specified RNA which is a normal intermediate in the synthesis of mature 4s RNA but which usually is so transient as to remain undetected. Two possible mechanisms consistent with the experimental data which would result in a diminished rate of manufacture of 'mature' tRNA are discussed below.

Accumulation of $4\frac{1}{2}$s RNA in infected cells may result from changes rendering the $4\frac{1}{2}$s to 4s conversion stage rate limiting. This may be due to a decrease in the level of activity of only one of the maturation enzymes (namely that involved in the conversion reaction). This, in turn, means that either the corresponding enzyme of the host is inhibited to a limited extent or its role assumed by a 'new', less efficient, enzyme. (i.e. infected cell

extracts contain (a) a partially effective host enzyme inhibitor or (b) a more effective inhibitor and a virus induced enzyme). Either possibility fits the data obtained by in vitro modification. Methylases, thiolases and tRNA modifying enzymes in general are obvious candidates. The appearance of specific undermodified tRNAs (which do not contain 2-methylthio, 6N (γ, γ dimethylallyl) adenylic acid) have been correlated with phage ϕ 80 infection.

Since the $4\frac{1}{2}$s RNA has been shown to be deficient in methyl groups and in view of the altered methylase pattern and the induction of virus specified methylase inhibitor in phage systems, the enzyme is most probably a methylase (Wainfan, 1968). Unlike the situation in Krebs II ascites cells, the configurational changes required to convert the open $4\frac{1}{2}$s configuration to the compact cloverleaf structure of 4s RNA cannot occur with unmodified tRNA precursor. Although the system outlined appears perfectly feasible, it should be noted that modification has been thought, in certain cases, to require as substrate RNA already in the cloverleaf structure, and that undermethylated tRNA accepts amino acids whereas configurationally incorrect tRNA species do not.

An alternative hypothesis would assume that in normal cells the machinery for tRNA production is membrane located and that

on pseudorabies virus infection solubilization of the requisite enzymic teams occurs, due to cellular disintegration. In other words, a rapid conversion of $4\frac{1}{2}$s to 4s is normally mediated by a reaction sequence controlled stereochemically by spatial enzymic arrangement (akin to the electron transport particles of mitochondria). In pseudorabies virus infected cells, the efficiency and rapidity of the process is severely diminished and this is reflected in the inability of infected cell extracts to catalyse 4s production. It must, of course, be assumed that extraction techniques do not destroy the functional enzymic-particles and that the system need not start from scratch but can accept intermediary species. The system seems quite reasonable especially since it is known that profound cytopathic changes are associated with pseudorabies virus infection and that pre-tRNAs are normally located in the endoplasmic reticulum.

In conclusion, therefore, it would appear that after pseudorabies virus infection of BHK21/13 cells, changes in the low molecular weight RNA components do occur. In particular, it would appear that normal tRNA maturation processes are slowed and intermediate products accumulate, that a new or altered 5s RNA species may be present and that a proportion of the 4s RNA

synthesised in infected cells is specified by the viral genome. The latter 4s RNA occupies 0.15% of the viral DNA and possesses all the characteristics of tRNA. At most, therefore, a viral DNA molecule would specify 4 tRNA molecules. Identification of this 4s RNA component in an aminoacylated form has not, however, been established in the tRNA populations so far examined. This failure is probably attributable to technical limitations. It would obviously be interesting to extend the investigations of aminoacyl-tRNA populations by utilizing other techniques, say, and to examine molecular hybridisation of radioactively labelled aminoacyl-tRNA with viral DNA.

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A P P E N D I X

INTRODUCTION

Elucidation of the effect of herpes simplex virus infection on the tRNA populations of the host BHK 21/13 cell, in furtherance of the data reviewed in the introductory section (p 63.), was undertaken. The procedure involving DEAE cellulose chromatography of T_1RNase digests of host and herpes simplex virus infected aminoacyl-tRNAs was used (Ishida & Miura, 1965; Herbert, Smith & Wilson, 1964). In particular, the arginyl-tRNA and the seryl-tRNA populations were examined. The exact methods used were identical to those already described for pseudorabies virus infected systems and the results obtained are outlined below.

RESULTS

Arginyl-tRNA in Herpes Simplex Virus Infected Cells

Fig. 39 shows the pattern obtained on DEAE cellulose chromatography of the components of a T_1RNase digest of a mixed tRNA preparation from uninfected and herpes simplex virus infected BHK 21/13 cells.

Figure 39.

Comparison of Arginyl-tRNA from Non-infected and Herpes Simplex Virus Infected BHK21/13 Cells by Co-chromatography of T_1RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 15 PFU per cell with herpes simplex virus. The preparations were separately charged with $[^{14}C]$ arginine (non-infected) and $[^{3}H]$ arginine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1RNase digestion products of 500 µg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ, for salt concentration and for total radioactivity associated with each isotope.

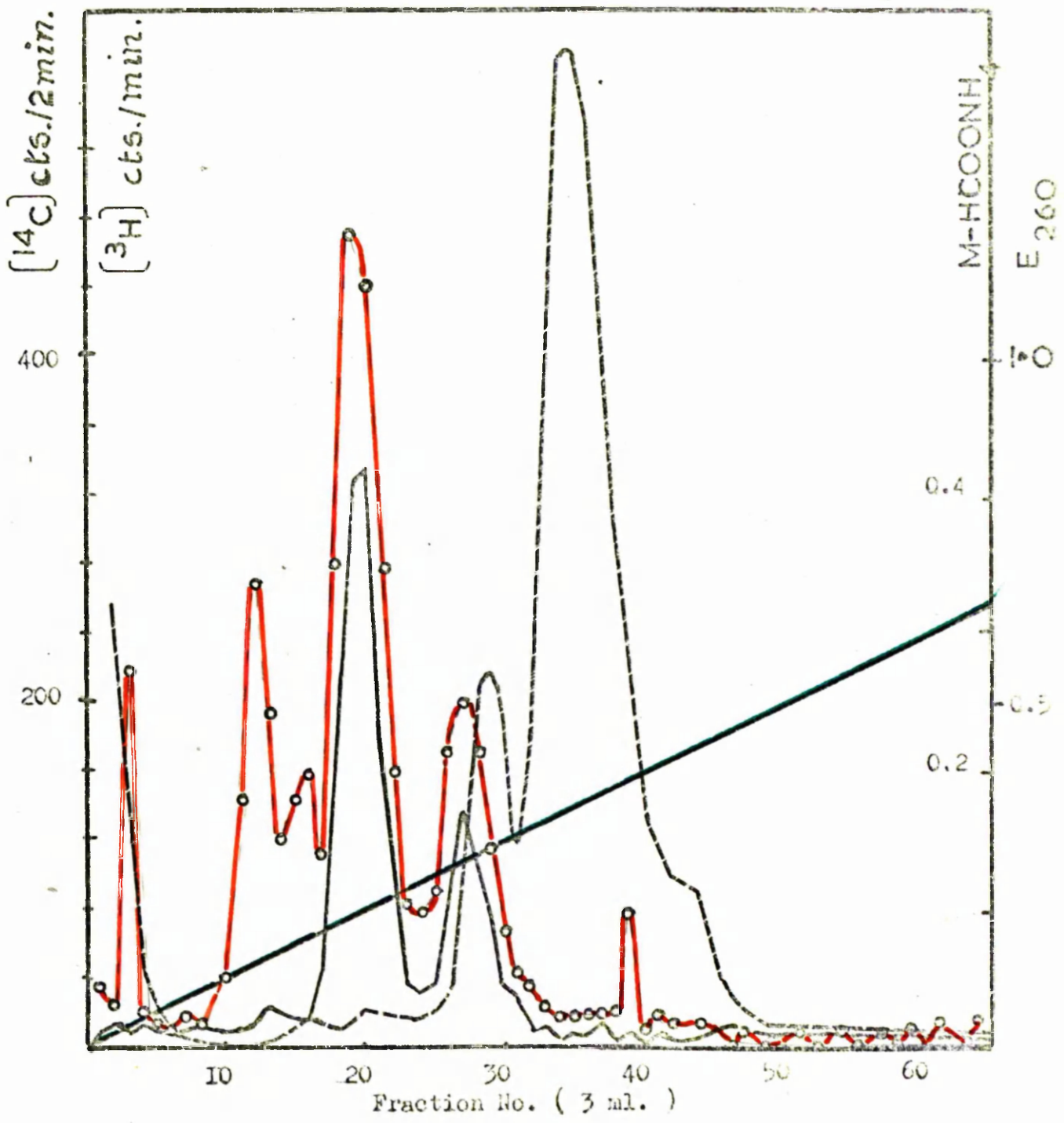
- - - E_{260}

——— $[^{3}H]$ cts./min./2 ml. of fraction.

——— $[^{14}C]$ cts./min./2 ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 39


[14C] cts./2min.
[3H] cts./min.
400
200
M-HCOONH4
E 260
0.4
0.2
10
20
30
40
50
60
Fraction No. (3 ml.)

The RNA had previously been labelled with [^{3}H] arginine (herpes simplex virus infected) and [^{14}C] arginine (non-infected). There are 4 peaks of [^{3}H] radioactivity eluting at 60 mM, 80 mM, 95 mM and 130 mM-ammonium formate and 2 peaks of [^{14}C] radioactivity at 95 mM and 130 mM. The appearance in the infected preparations of 2 peaks, at 60 mM and 80 mM, which contain only [^{3}H] counts indicates that at least one, and probably two, new species of arginyl-tRNA are present in herpes virus infected cells. It can also be concluded, from the molarity of elution, that the terminal nucleotide sequences at the 3' end of these species differ from those of the cellular arginyl-tRNA in that the first guanosine residues are nearer to the $pCpCpA$ terminal end. The background corrected [^{3}H] counts in the 4 peaks are 362: 190: 886: 438: respectively, while the [^{14}C] counts are 600: 270. This suggests that about 30% of the arginyl-tRNA in herpes virus infected cells at 6.5 hours post infection, is virus induced. Calculation of the ratio of total radioactivity in the peak centred at 95 mM to that in the peak centred at 130 mM gives a value of 2.0/1 in the case of the herpes simplex infected preparation and 2.2/1 in the case of the control.

These values appear to be the same within the limits of experimental error. It would, therefore, appear that herpes simplex virus infection neither induces any new arginyl-tRNA species with a terminal base sequence identical to that of host specific arginyl-tRNA, nor selectively stimulates the production of a single species of the host arginyl-tRNA. The alteration in the population of arginyl-tRNA species in infected cells is, therefore, completely attributable to the synthesis of 1 or 2 species of tRNA, the base sequence of which does not correspond with that of the uninfected host cell species.

Seryl-tRNA in Herpes Simplex Virus Infected Cells

Illustrated in Fig. 40 is the chromatographic profile, on DEAE cellulose, of oligonucleotides produced by T_1RNase digestion of mixedly labelled preparations of seryl-tRNA from uninfected $[^3H]$ and herpes virus infected $[^{14}C]$ BHK 21/13 cells. There are 3 peaks in the infected preparation eluting at 50 mM, 75 mM or 100 mM-ammonium formate, the total number of $[^{14}C]$ disintegrations in each peak being 21,460: 2,840: 2,660. The control preparation has a single major peak eluting at 50 mM-ammonium formate.

Figure 40.

Comparison of Seryl-tRNA from Non-infected and Herpes Simplex Virus Infected BHK21/13 Cells by Co-chromatography of T_1RNase Digestion Products on DEAE Cellulose.

sRNA was extracted from actively growing non-infected BHK21/13 cells and from cells 6.5 hrs. P.I. at a multiplicity of exposure of 15 PFU per cell with herpes simplex virus. The preparations were separately charged with $[^3H]$ serine (non-infected) and $[^{14}C]$ serine (virus infected). After deproteinisation, the isolated aminoacyl-tRNAs were mixed and the T_1RNase digestion products of 600 µg. of the mixture deproteinised and chromatographed at 4° on a 1 x 5 cm. column of DEAE cellulose. A linear gradient of ammonium formate, pH 5.5 was used for elution and 3 ml. fractions were collected. Each fraction was monitored for extinction at 260 mµ , for salt concentration and for total radioactivity associated with each isotope.

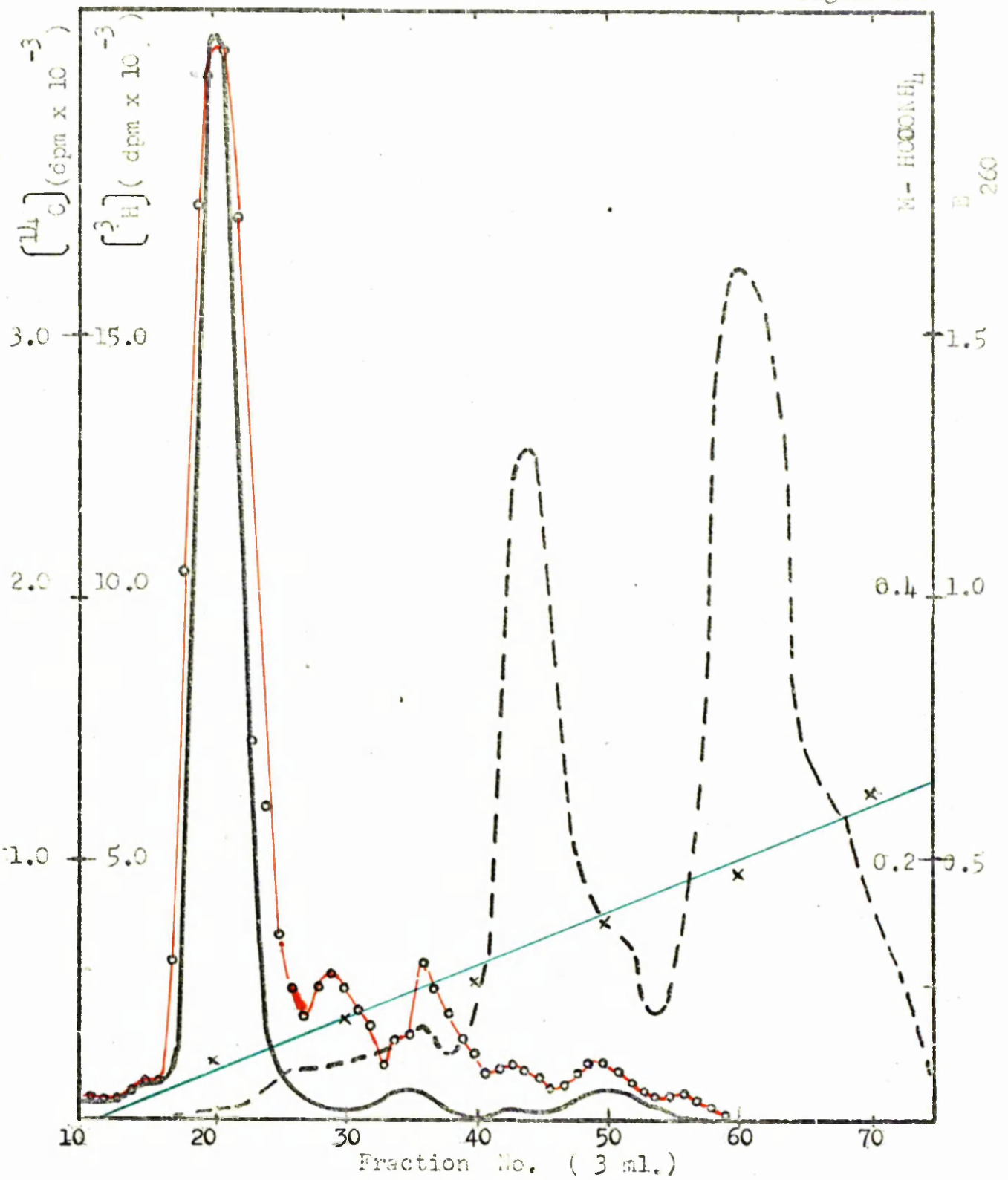
--- E_{260}

——— $[^3H]$ disintegrations/min. /2 ml. of fraction.

——— $[^{14}C]$ disintegrations/min. /2 ml. of fraction.

——— Ammonium formate concentration (expressed as molarity).

Figure 40.


$[^{14}C]$ (dpm x 10^{-3})
$[^{3}H]$ (dpm x 10^{-3})
M- $HCOONH_4$
E_{260}
3.0
15.0
2.0
10.0
1.0
5.0
1.5
0.4
1.0
0.2
0.5
10
20
30
40
50
60
70
Fraction No. (3 ml.)

From the appearance, magnitude and elution position of the peaks at 75 and 100 mM-ammonium formate, it can be deduced that 20% of the seryl-tRNA present in cells infected with herpes virus for 6.5 hours is attributable to 2 new species of tRNA, the terminal nucleotide sequences of which differ from those in the normal host population in that guanosine residues are located further from the $p^{C}p^{C}p^{A}$ terminus.

This difference in the elution profiles of seryl-tRNA obtained from non-infected and herpes virus infected BHK 21/13 cells was confirmed when these two types of tRNA were prepared a second time in parallel, by identical procedures, and labelled in the reverse sense to the above before chromatography.

DISCUSSION

The results obtained, therefore, demonstrate differences in the populations of arginyl-tRNA and seryl-tRNA as extracted pre and post virus infection. Indications are, that in infected cells, one and perhaps two additional species of arginyl-tRNA (equivalent to 30% of arginyl-tRNA in infected cells) and two additional species of seryl-tRNA are present.

These molecules differ in base sequence, at the 3' end of the molecule, from normal components of the arginyl-tRNA and seryl-tRNA populations of non-infected cells respectively. The first guanosine residue from the 3' end in either of the two new arginyl-tRNAs is positioned nearer to that end than normal. The reverse situation pertains for the new seryl-tRNA, i.e., the first guanosine residue occurs nearer to the 5' end than normal. Singly, these results could be attributed to any of the following modifications of existing tRNA molecules:- (1) the conversion of a guanosine base to a form not recognised by T_1RNase (e.g. by methylation); (2) the alteration of another base to one mimicking-guanosine in susceptibility to T_1RNase activity; (3) dimerisation of tRNA molecules (Loehr & Keller, 1968). Taken together, however, such possibilities seemed less feasible, especially since no modified bases have been detected at the 3' end of any of the sequenced tRNAs (Zauchau Dutting & Feldman, 1966; Staehelin et al, 1968; Raj-Bhandary et al, 1967; Holley et al, 1965). In the absence of other possible experimental artefacts, it can,

therefore, be postulated that the two additional arginyl species and the two additional seryl species are synthesised de novo in herpes simplex infected cells, or are attributable to arginine or serine attachment to tRNAs normally loaded with other amino acids. The latter explanation appears to contradict the requirements for accurate m RNA translation and has therefore been eliminated. The results obtained, when taken in conjunction with the hybridisation data published, strongly suggest that the new tRNAs are virus specified and not due to virus induced modification of host specified tRNA. The additional arginyl-oligonucleotides detected probably are derived from the arginyl-tRNA from herpes simplex infected cells which is maximally delayed on MAK. Since experimental artefacts in the T_1RNase system are so much fewer than those found with MAK, possible explanations of the nature of the new tRNAs are narrowed (aggregates, mathylation, thiolation, configurational alterations) and the additional species of tRNA in herpes simplex infected cells interpreted as new virus specified arginyl or seryl-tRNAs.